

**REPRODUCTION OF *ZEBRASOMA FLAVESCENS*: OOCYTE  
MATURATION, SPAWNING PATTERNS, AND AN ESTIMATE OF  
REPRODUCTIVE POTENTIAL FOR FEMALE YELLOW TANG IN  
HAWAI'I**

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## GENERAL INTRODUCTION

*Zebrasoma flavescens* is one of 80 species within the surgeonfish family Acanthuridae (Nelson, 2006). Acanthurids are gonochoristic and primarily herbivorous, and are found in coral reef habitats in tropical and subtropical seas, although they are absent in the Mediterranean (Nelson, 2006). *Zebrasoma flavescens* (the yellow tang) and several other surgeonfish species are common on most reefs in the Hawai'ian islands, including an exceptionally large population along the Kona coast of the Big Island of Hawai'i. In fact, local mythology suggests that the nickname 'Gold Coast' is in reference to the many golden-colored fishes visible in the shallow waters of Kona, thanks to the prevalence of yellow tangs.

Yellow tang provide 70.5% of the total catch for the aquarium trade in the state of Hawai'i, and the majority of these fish are taken from the Kona (west) side of Hawai'i island (Hawai'i Division of Aquatic Resources, unpublished data, FY 2006). At the urging of local interests, whose intentions were to protect coral reef fish targeted for the aquarium trade, the state legislature established a network of nine separate Fish Replenishment Areas (FRAs) on the Kona coast of Hawai'i in 2000. Currently, these protected areas, where aquarium fishing is prohibited, encompass over 30% of the total western coastline. Since the inception of the FRA network, the Hawai'i Division of Aquatic Resources (HDAR) has been charged with maintaining populations of popular reef species, including the yellow tang, at levels that satisfy fishing interests, the burgeoning snorkel/scuba diving industry, and local conservationists.

Information regarding the basic reproductive biology of a species can contribute to developing appropriate fishing regulations in terms of both location and season. The purpose of this research was to elucidate the timing and seasonality of yellow tang spawning along the Kona coast, and to use this information to estimate the reproductive potential of adult females within the population. Specific aims of this study were

achieved in the order presented, and have been divided into three separate chapters within this thesis.

The first objective of this research was to form a more complete understanding of the reproductive process in yellow tang by analysis of oocyte maturation. Previous studies on this species in the Hawai'ian Islands have used the gonadosomatic index (GSI) as an indicator of reproductive output (Lobel, 1989; C.W. Laidley, pers. comm.). However, discussions in the literature have implied that GSI alone may not accurately predict spawning output for certain fish species (West, 1990; Stequert *et al.*, 2003; Kritzer, 2004). Although increased GSI may sometimes serve as a reliable indicator of spawning, the most comprehensive investigations of fish reproductive patterns relate GSI to actual ovarian and oocyte state before making this assumption (Jons and Miranda, 1997; Somarakis *et al.*, 2004). Most commonly, this is achieved by means of a histological assessment of ovarian and oocyte maturation. To date, only one other species of acanthurid (*Acanthurus nigrofuscus*) has been investigated histologically in reference to reproduction (Fishelson *et al.*, 1987). A histological investigation of *Z. flavescens* would make a major contribution to understanding reproductive characteristics of this abundant and widespread reef fish family.

A thorough study of oocyte maturation patterns in yellow tang, including staging of oocytes throughout development and over the course the diel cycle, was undertaken as the first objective in Chapter 1 of this thesis. The information obtained was subsequently used to investigate monthly and seasonal spawning cycles of the species, reported in later chapters of the thesis.

The second objective of this research was to determine the annual peak of reproductive output for a particular population of yellow tang on the Kona Coast.

Knowledge concerning spawning seasonality is an important aspect of food fish population management, as it can influence regulations such as size limits on catch and seasonal closures (Williams and Blood, 2003; Kritzer, 2004; Williams and Shertzer, 2005). It follows that reproductive seasonality data should also be taken into consideration in management of fish species targeted by the aquarium trade such as *Z. flavescens*.

In Chapter 2 of this thesis, the spawning seasonality of *Z. flavescens* was investigated by sampling over the course of one calendar year (May 2006-April 2007). Adult females were chosen as the subject of study because the most obvious characteristics of reproductive output (*i.e.*, changes in gonad weight and gamete production) are both more evident and more easily quantifiable in females than in males. A lunar cycle of spawning was hypothesized due to a perceived increase in both GSI and batch fecundity of females sampled around the full moon in preliminary studies, as well as in the observed spawning patterns of captive fish (C.W. Laidley, unpublished data), and was therefore also investigated.

The last objective of this work, addressed in Chapter 3, was to estimate total annual reproductive output for female yellow tang in Kona, Hawai'i. This estimate is intended to assist managers in calculations of the standing stock biomass required to maintain population size in the face of increasing harvesting rates. Other populations under intense fishing pressure have prompted similar studies that use reproductive output estimates in large-scale stock assessment models (for review, see Stratoudakis *et al.* (2006)). A size-fecundity relationship for females is usually incorporated into such models, as the number of eggs produced by a given female can vary with weight or standard length of the individual (reviewed by Birkeland and Dayton (2005)). For this reason, the relationship between size and fecundity of yellow tang was also addressed

in this final chapter. Previously documented methods for calculating annual fecundity of multiple-spawning fishes were not applicable to this study. (A detailed treatment of this topic is provided in Chapter 3.) Therefore, a regression model based on data collected during the year of sampling was used in the estimation.

## **CHAPTER 1.**

### **A histological investigation of oocyte development and post-ovulatory follicle (POF) degeneration in *Zebrasoma flavescens*, the yellow tang**

#### **ABSTRACT**

The purpose of this work was to investigate ovarian composition and to describe oocyte stages of development in *Zebrasoma flavescens*, the yellow tang. A histological investigation of ovaries was carried out on fish sampled from a population off the Kona coast of Hawai'i during 2005-2007. Oocytes were divided into 8 stages of growth: primary growth stage (I) [subdivided into chromatin nucleolar (Ia) and perinucleolar (Ib) stages], cortical alveolar stage (II), vitellogenic stage (III) [subdivided into early (IIIa), middle (IIIb), and late (IIIc) vitellogenic stages], and mature stage (IV) [subdivided into maturing (IVa) and hydrated (IVb) stages]. Each stage was pictorially documented and described according to accepted terminology in the literature. Post-ovulatory follicle (POF) degradation in captive fish was documented at 6-hour intervals for 48 hours, and distinctions between new and 24-hour old POFs were established. These histological findings have established that this species is a multiple-spawning fish with asynchronous oocyte development and indeterminate fecundity (as defined by Hunter and Macewicz (1985)). A diel pattern of spawning was recognized, and repeat spawning at least for two successive days was indicated for female *Z. flavescens*.

#### **INTRODUCTION**

Staging of oocytes by means of histological methods has been helpful in determining the time necessary for oocyte maturation and time-to-spawning in many fishes (DeMartini and Fountain, 1981; Matsuyama *et al.*, 1988; Scott *et al.*, 1993; Coward and Bromage, 1998). For many species of interest, studies have also investigated the relationship of the gonadosomatic index (an expression of gonad weight as a fraction of

non-gonadal body weight, or GSI) to histologically determined germ cell stage, in order to more clearly define the maturational state of the individuals examined (*e.g.*, Somarakis *et al.*, 2004). For this thesis, histological assessment of oocyte stages, fecundity measurements and calculation of GSI were used in the investigation of female yellow tang reproduction. Prior to incorporation of the latter methods (in Chapters 2 and 3), however, the basic process of yellow tang oocyte maturation was first assessed using light microscopy of histologically prepared ovarian tissue, presented here in Chapter 1.

Histological investigations of ovarian development in fishes have provided researchers with highly detailed information concerning the morphology and functional processes of egg maturation in the species of interest (Hunter and Macewicz, 1985; Selman *et al.*, 1993). Other, less time-consuming (and less specific) methods of ovarian maturity assessment include macroscopic evaluation of ovaries (such as assessment of ovarian maturity based on size, weight or appearance of tissue), gross measurement of oocyte sizes, and gonad-weight indices. However, to attain a thorough understanding of the oocyte maturation process and its relationship to spawning events in the reproductive assessment of any fish species, histology is the most accurate tool available and can produce a more complete picture of reproduction, when compared to gonad-weight indices (such as GSI) or macroscopic ovarian assessment alone (Kritzer, 2004).

The first part of this chapter documents and describes the stages of yellow tang oocyte development based on morphological criteria observed with light microscopy as per previous studies on other teleost fishes (Selman *et al.*, 1993; Tyler and Sumpter, 1996; Rideout *et al.*, 1999; Ravaglia and Maggese, 2002). Classical studies have established a staging regime and a vocabulary to describe the process of oocyte maturation for hundreds of fish species (*e.g.*, Van den Hurk and Peute, 1979; DeVlaming, 1983; Wallace and Selman, 1981; Selman *et al.*, 1988; Selman and Wallace, 1989). The basic mechanisms of oocyte growth are similar among most teleosts, except for variation in timing and recruitment between stages (Wallace and Selman, 1981). Current descriptive

terminology has been used in this chapter, following the language of Patino and Sullivan (2002) and Maack and Segner (2003). Once staged, oocyte populations in ovaries of *Z. flavescens* sampled at different times of day were examined, and inference concerning the rate of egg batch production and time of release was possible.

The second part of this chapter documents and describes specific ovarian structures, post-ovulatory follicles (POFs), observed within the ovary after egg release. During development and until ovulation, the oocyte is surrounded by a multi-layer follicle, consisting of an inner layer of granulosa cells with an associated basal lamina and an outer layer of theca cells, also associated with meso-epithelial cells and blood capillaries (Tyler and Sumpter, 1996 and illustrated in Figure 1-1). Upon ovulation, the oocyte is released from these surrounding follicle cells into the lumen of the ovary, from where it will be expelled via smooth muscle contractions of the ovarian wall during spawning. The follicular assemblage collapses away from the opening formed by the release of the oocyte and folds in onto itself, creating a POF, an example of which can also be seen in Figure 1-1. In many studied teleost species, (including, but not limited to, *Sardinella brasiliensis*, *Pagrus major*, *Tilapia zilli*, and *Lutjanus campechanus*,

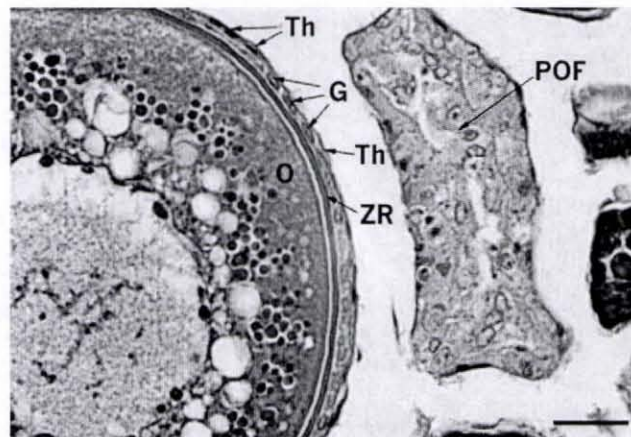


Figure 1-1. A multi-layer follicle, composed of theca and granulosa cell layers, surrounding a vitellogenic oocyte (stage IIIb) in a *Z. flavescens* ovary. Th = theca cells, G = granulosa cells, ZR = zona radiata, O = oocyte cytoplasm, POF = post ovulatory follicle. Scale bar 20  $\mu$ m.

as investigated by Isaac-Nahum *et al.* (1988), Matsuyama *et al.* (1988) Coward and Bromage (1998), and Jackson *et al.* (2006)), POFs are broken down by macrophage activity within a period of less than 2 days. Therefore, the presence of POFs is generally taken to be indicative of spawning within a relatively recent period of time for most fish species (Hunter and Macewicz, 1985; Selman and Wallace, 1989), with an inverse correlation between water temperature at spawning and length of time to follicular degeneration (Hunter and Macewicz, 1985). In the current study, it was hypothesized that in a tropical species such as *Z. flavescens*, an estimation of time-to-breakdown of POFs would offer some indication as to the frequency of spawning in the population investigated.

## **MATERIALS AND METHODS**

### ***Fish collection***

(For details pertaining to the sites of collection, see Chapter 2.)

Female adult *Z. flavescens* were sampled from a wild population by SCUBA divers using three-pronged spears. At least one field collection occurred on the approximate hour between 0900 and 1700 hrs during the summer of 2005. Fish collected in 2005 were used only for the histological investigation of diel cycling of egg maturation in this population, as presented in this chapter.

All collections of adult females during 2006 and 2007 were performed between 1500 and 1730 hrs. Fish sampled in 2006 and 2007 were also used in spawning seasonality investigations further detailed in Chapters 2 and 3.

### ***Fish sex identification***

A consistent difference in the genital openings of male and female *Z. flavescens* was noted early in the study. After standardizing dissection techniques, 23 adult fish from the May 14, 2006 collection were examined prior to dissection, and identified as



having either a female or a male genital opening as illustrated in Figure 1-2. Subsequent dissection and macroscopic examination of the gonads by eye established a 100% correlation between the initial identification of sex and the actual sex of the individuals in this collection, and in all subsequent collections of fish in which this protocol was followed.

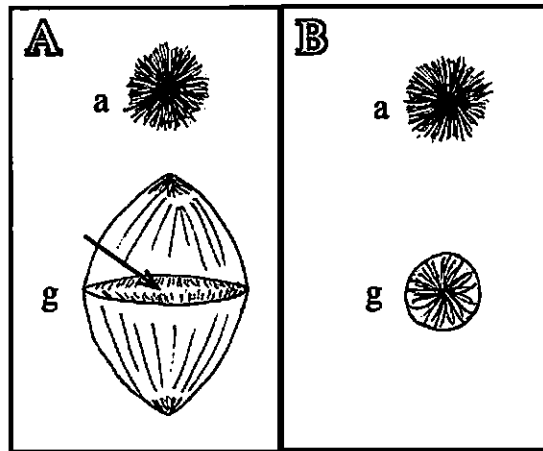


Figure 1-2. Diagrams of (A) adult female and (B) adult male genital openings of *Z. flavescens* drawn to the same scale. The (a) anus is anterior to the (g) genital opening in both sexes. The arrow denotes the opening (slit) from which eggs are released. Sperm are released from the center of the male genital opening.

#### ***Post-ovulatory follicle (POF) holding experiments***

Three holding experiments were carried out on June 10, 2006, on July 9, 2006 and on April 3, 2007. In each instance the following protocol was used.

Three divers on SCUBA herded adult fish into a barrier net in a shallow water location (approximately 8-10 m in depth) within the sampling site. All fish captured in the net were sexed under water by the divers by noting the shape of the genital opening (Figures 1-2 and 1-3), and all females were maintained in a 35-gallon underwater plastic container with lid and 1-cm diameter holes until the end of the dive. The underwater container was then attached to a line suspended from the boat above, and was subsequently pulled

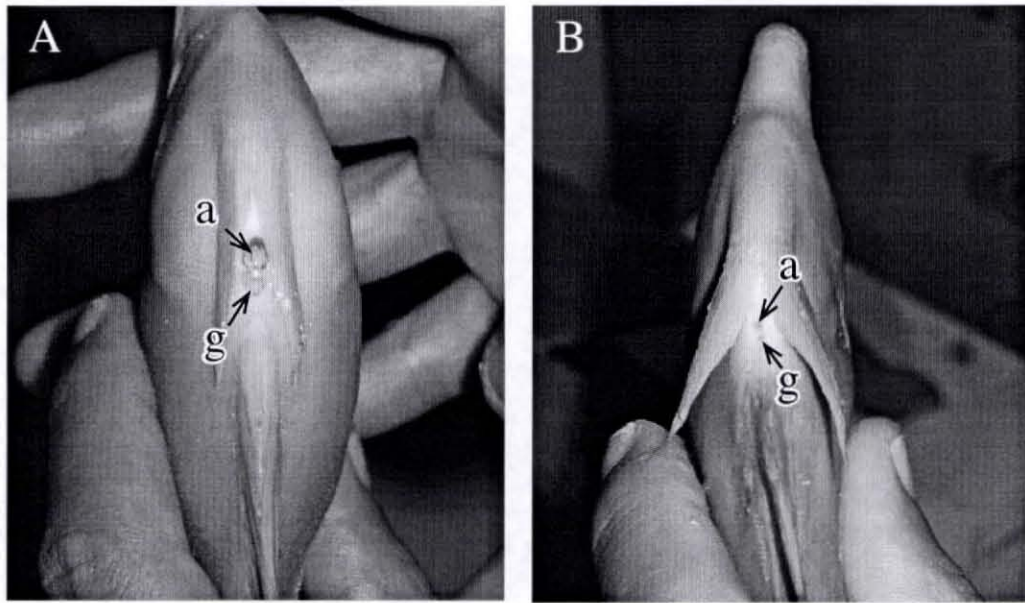


Figure 1-3. Photographs of genital and anal openings of (A) adult female and (B) adult male *Z. flavescens*. The (a) anus is anterior to the (g) genital opening. Females have a much larger genital opening than males. This particular female has an enlarged anal opening, probably due to internal pressures on the gut contents from an inflated swim bladder.

up to the surface in small vertical increments over a period of 45 – 60 minutes to allow fish to acclimate to changes in pressure. Once on the surface, females were placed in a large bucket filled with seawater and lightly massaged along the belly. The presence of ovulated eggs emitted from the cloacal opening was taken to be evidence of ‘readiness to spawn’ for that evening. All females with expressed eggs were kept, and all other fish were released.

In total, 30 ready-to-spawn females were then transported to a land-based holding facility and maintained in either aerated 25-gallon square plastic containers holding sea water (fresh sea water was periodically added by hand), or in sea water flow-through tanks for up to 48 hours. (Tanks were circular, 1.5 m in diameter by 1 m deep, with surface seawater filtered by a 5µm cartridge at 6 L/min continuous flow.) Between four and six fish were housed per tank or container. All fish were kept outdoors on a natural photoperiod. Fish were humanely euthanized by decapitation (as per University of

Hawai'i IACUC protocols) at pre-determined time points of 6, 12, 18, 24, 30, 36, 42 and 48 hours following the assumed time of spawning, ½ hour prior to sunset (Walsh, 1984). Four fish were euthanized at each time point except at time points 18 and 48 hours; due to the size of the total collection (30 fish), only three fish each were available for the 18-hour and 48-hour time points.

### ***Fish processing***

For preservation of tissue, fish were placed immediately on ice after spearing or decapitation, and gonad dissections commenced within 12 hours. Each fish was weighed, sexed, and measured for total length (TL) and standard length (SL). TL was defined as the length between the tip of the snout and the posterior rim of the caudal fin; SL was defined as the length between the tip of the snout and the posterior end of the midlateral portion of the hypural plate. All gonads were removed in their entirety from the body cavity, blotted with a paper towel and weighed, and placed in Dietrich's fixative (600 ml distilled water; 300 ml 95% ethanol; 100 ml 37% formaldehyde; 20 ml glacial acetic acid) for histological processing.

### ***Histology***

Ovaries processed for histology were maintained for at least one month in Dietrich's fixative, then embedded in either paraffin or JB-4 plastic and sectioned at 6 µm, for both embedding media. Paraffin samples were stained with commercially obtained Harris haematoxylin (Sigma-Aldrich) and eosin Y (Sigma-Aldrich), and plastic sections were stained with o-toluidine (Sigma-Aldrich) according to standard protocols.

### ***Oocyte measurements***

Measurements of cell diameters on histological slides were taken either by using a calibrated ocular micrometer or by using QCapturePro software (available from Q Imaging) calibrated to the microscope magnification used. Only oocytes that were sliced

through the nucleus were measured. In relation to the overall size of an oocyte, the nuclear region is large, and is therefore likely to be sliced at angles other than through its center, making the measurement of cross-sectional oocyte diameters of fish analyzed in such a way subject to a recognized margin of error. Although the probability of slicing through the center of the nucleus in a given section is directly related to the diameter of the nucleus itself, this technique was determined to be the most efficient of the techniques previously established in the literature (see West (1990) for review), even though it resulted in an inexact measurement of cross-sectional diameter of every stage analyzed. Oocyte diameters taken by this method are presented in Table 1-1.

Oocyte diameters were measured at a randomly chosen angle, either perpendicular or parallel to the field of view (West, 1990). Diameters of fresh hydrated oocytes were measured at 10x magnification using an ocular micrometer, also at an angle either perpendicular or parallel to the field of view.

#### *Measurements of POFs*

Using QCapturePro software, it was possible to draw a line at the widest part of each POF (the X-axis), inclusive of all tissue. A second line was drawn perpendicular to the first, also inclusive of all POF tissue, and was treated as the Y-axis. The two axes were equated to length and width of an imaginary rectangle, and were therefore multiplied to determine the approximate area occupied by the POF within the ovary.

## RESULTS

### *Staging of oocytes*

Because oocytes in all teleosts undergo the same basic pattern of maturation and differentiation – primary oocyte growth, cortical alveolar stage, vitellogenesis, maturation and ovulation (Tyler and Sumpter, 1996; Patino *et al.*, 2003) - the division of *Z. flavescens* oocyte maturation into stages was relatively uncomplicated. In this study, eight stages of oocyte development were distinguished based on morphological

Table 1-1. Summary of diameter size ranges (in microns) for each stage of oocyte maturation in *Z. flavescens*. Oocyte diameters were measured with qCapture Pro software at 4x, 10x or 40x magnification using an Olympus cx31 compound microscope. Hydrated oocytes were measured fresh, post-ovulation (see text for explanation).

<i>Stage</i>	<i>Size range (mean diameter <math>\pm</math> std. dev.)</i>	<i>No. oocytes measured (N)</i>
I. a) Chromatin nucleolar	10-55 (25.6 $\pm$ 8.6)	100
I. b) Perinucleolar	23-69 (43.9 $\pm$ 9.3)	99
II. Cortical alveolar	42-136 (83.2 $\pm$ 23.1)	149
III. a) Early vitellogenic	94-205 (137.1 $\pm$ 19.8)	93
III. b) Middle vitellogenic	135-242 (178.9 $\pm$ 25.3)	114
III. c) Late vitellogenic	202-318 (271.1 $\pm$ 26.8)	105
IV. a) Maturing	340-596 (452 $\pm$ 61.0)	137
IV. b) Hydrated	500-900 (734.5 $\pm$ 63.2)	156

characteristics, staining affinity, and size (diameter): primary growth stage (I) [subdivided into chromatin nucleolar (Ia) and perinucleolar (Ib) stages], cortical alveolar stage (II), vitellogenic stage (III) [subdivided into early (IIIa), middle (IIIb), and late (IIIc) vitellogenic stages], and mature stage (IV) [subdivided into maturing (IVa) and hydrated (IVb) stages]. A description and pictorial documentation of each stage follows.

### **I. Primary oocyte growth stages**

During the primary growth stage of *Z. flavescens* oocytes, follicular cells surrounding the developing oocyte were present, though indistinct, and were therefore not described in detail until they became more obvious in later stages of maturation. Primary oocytes (stages Ia and Ib) were observed in all ovaries examined.

#### *Ia) Chromatin nucleolar stage*

*Size range: 10-55 $\mu$ m (mean diameter = 25.6  $\pm$  8.6  $\mu$ m)*

Oocytes contained a central, lightly stained nucleus (or germinal vesicle (GV) as defined



by Ransom, 1867) with a single, large nucleolus (Figure 1-4). The nucleus was basophilic and large relative to cytoplasm; the cytoplasm was grainy and uniformly dark, with a more intensely stained nucleoplasm. Oil droplets (which are involved in the formation of the lipid globule of fully developed eggs (DeVlaming, 1983)) occurred in the cytoplasm, even at this early stage.

#### Ib) Perinucleolar stage

Size range: 23-69  $\mu\text{m}$  (mean diameter =  $43.9 \pm 9.3 \mu\text{m}$ )

The perinucleolar stage oocyte was increased in overall size compared to the chromatin nucleolar stage oocyte (Figure 1-4). The GV was also increased in size, and took on an ovoid shape in the later perinucleolar stage. The GV:cytoplasm ratio was still high, and multiple nucleoli adjoining the nuclear envelope appeared. Often, one nucleolus remained large (close in size to the chromatin nucleolar stage nucleolus), while several

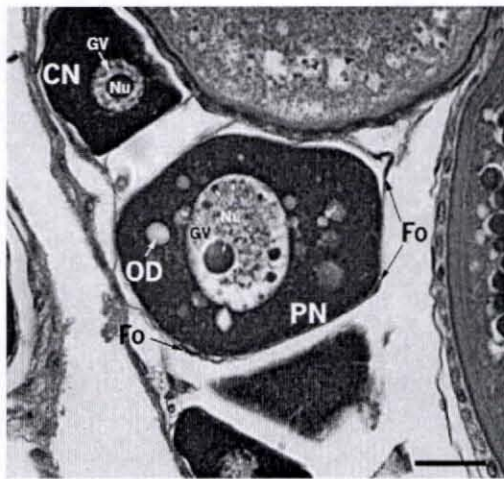


Figure 1-4. Primary oocytes in a *Z. flavescens* ovary. CN = chromatin nucleolar stage oocyte, Fo = follicle cells, GV = germinal vesicle, Nu = nucleolus, PN = perinucleolar stage oocyte, OD = oil droplet. Scale bar 20  $\mu\text{m}$ .

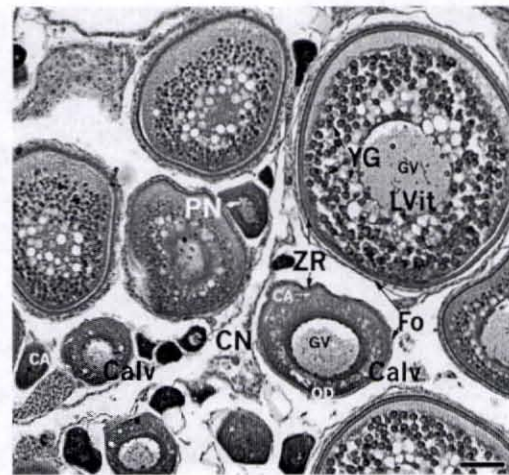


Figure 1-5. Cortical alveolar stage oocytes in a *Z. flavescens* ovary. CA = cortical alveoli, Calv = cortical alveolar stage oocyte, CN = chromatin nucleolar stage oocyte, Fo = follicle cells, GV = germinal vesicle, LVit = late vitellogenic stage oocyte, PN = perinucleolar stage oocyte, OD = oil droplet, YG = yolk granule, ZR = zona radiata. Scale bar 50  $\mu\text{m}$ .

other smaller nucleoli, recent products of division from the original nucleolus, could be observed nearby within the GV. The oocyte cytoplasm was still stained uniformly dark with haemotoxylin. Follicle cells were evident at 40x magnification as a thin line surrounding the oocyte, and oil droplets were often present in the cytoplasm of perinucleolar oocytes (Figure 1-4).

## II. Cortical alveolar stage

*Size range: 42-136 $\mu$ m (mean diameter = 83.2  $\pm$  23.1  $\mu$ m)*

Oocytes more than doubled in size during stage II. This stage began with the first appearance of cortical alveoli – vesicles that release their contents into the perivitelline space between the oocyte and the overlying acellular vitelline envelope post-fertilization (Selman *et al.*, 1988) – and ended with the appearance of yolk granules. Cortical alveoli and oil droplets appeared within the cytoplasm in a ring formation surrounding the GV (Figure 1-5). (Selman and Wallace (1989) and Ravaglia and Maggese (2002) note that oil droplets can be difficult to distinguish from cortical alveoli depending on the nature of histological preparation.) In *Z. flavescens* oocytes, the distinction between the two cell types was made based on size (oil droplets were larger) and position within the oocyte (cortical alveoli were more likely to be seen toward the periphery of the cytoplasm), since staining specifically for cortical alveoli was not performed.)

Toward the end of stage II, the zona radiata (ZR) - the acellular vitelline envelope that develops around the outside of the oocyte at the primary growth stage, and continues to widen and differentiate throughout oocyte development, finally creating the chorion of the mature ovum (reviewed by Tyler and Sumpter (1996)) - became distinct as a thin line surrounding the oocyte. Throughout stage II, the oocyte increased in size, oil droplets increased in size and number, and evidence of follicular cells surrounding the cytoplasm became apparent by the end of this stage (Figure 1-5). Cortical alveolar stage oocytes were observed in all ovaries examined.

### III. Vitellogenic stage

The vitellogenic stage of *Z. flavescens* has been subdivided into three separate stages, based on distinct morphological characteristics of oocytes undergoing the vitellogenic process. Vitellogenic stage oocytes (stages IIIa, IIIb, and IIIc) were observed in all ovaries examined.

#### IIIa) Early vitellogenesis

*Size range: 94-205µm (mean diameter = 137.1 ± 19.8 µm)*

The onset of vitellogenesis (as reviewed by Tyler and Sumpter (1996)) was characterized by the addition of yolk granules to the cytoplasm, along with the aforementioned cortical alveoli and oil droplets. Oil droplets of various sizes were more centrally located in a ring formation around the outside of the GV, with very small yolk granules, which stained darker with eosin, appearing in the outer cytoplasm (Figure 1-6). Cortical alveoli were observed at the periphery of the cytoplasm. The ZR was distinct, with a width of about 1 µm (Table 1-2). A distinct, multi-layer follicle, composed of a squamous thecal cell layer, a basal membrane, and a granulosa cell layer, (Tyler and Sumpter, 1996), was evident in all vitellogenic oocytes (Figures 1-1 and 1-6), although the basal membrane was not visible using light microscopy.

Table 1-2. Differences in cellular components of oocytes in the vitellogenic stage (III) in *Z. flavescens*. Ovaries of 7 Group M fish were used in the compilation of these data. Two of the largest oil droplets and two of the largest yolk globules were measured for each of the twenty oocytes analyzed (N). GV:C = germinal vesicle to cytoplasm ratio. ZR = zona radiata. All measurements are in microns in the format of mean ± standard deviation.

<i>Oocyte stage</i>	<i>Oil droplet diameter</i>	<i>ZR width</i>	<i>Yolk granule diameter</i>	<i>GV diameter</i>	<i>GV:C</i>	<i>N</i>
IIIa	9.6 ± 2.0	1.7 ± 0.5	2.2 ± 0.6	62.2 ± 17.2	0.42 ± 0.08	20
IIIb	13.8 ± 3.4	3.6 ± 1.0	6.6 ± 1.7	73.2 ± 16.0	0.36 ± 0.07	20
IIIc	19.9 ± 4.6	7.4 ± 2.3	12.7 ± 2.5	89.3 ± 14.7	0.31 ± 0.05	20



The GV in early vitellogenic oocytes changed from a circular to an ovoid shape, with a 60-90  $\mu\text{m}$  lengthwise dimension, and remained about that size until final maturation (Table 1-2). The GV:cytoplasm ratio in the early stage of vitellogenesis was approximately 0.4 (Table 1-2).

### IIIb) Middle vitellogenesis

Size range: 135-242  $\mu\text{m}$  (mean diameter =  $178.9 \pm 25.3 \mu\text{m}$ )

As vitellogenesis progressed, yolk granules increased in number and in size and began to fill the surrounding cytoplasm, even as the oocyte itself increased in size (Figure 1-6). In the middle vitellogenic stage, the yolk particles were about twice the size of those found in the early vitellogenic stage, and had begun to form a ring around the oil droplets, which in turn surrounded the GV (Figure 1-6). Haematoxylin-stained cytoplasm was still visible, especially at the periphery of the oocyte. The ZR stained darkly and widened to

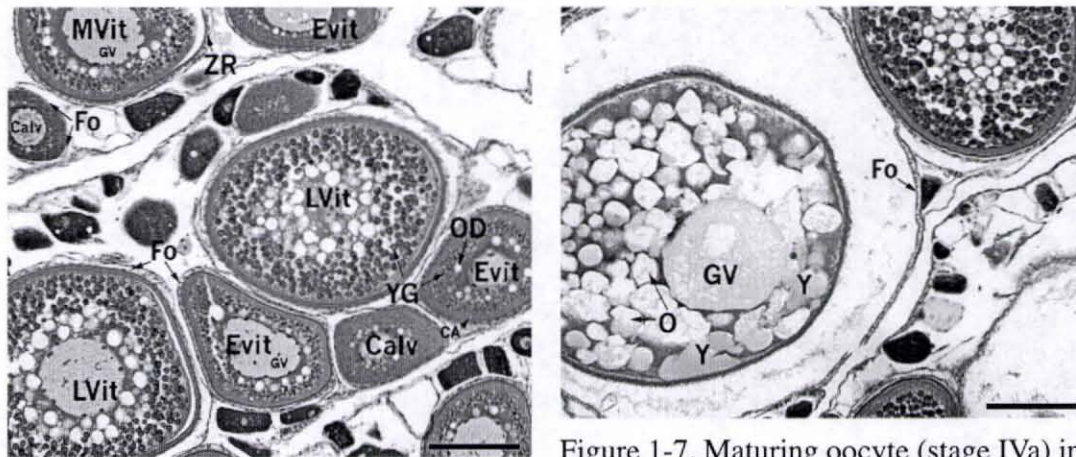


Figure 1-6. Early, middle and late vitellogenic stage oocytes (stage III) in a *Z. flavescens* ovary. CA = cortical aveoli, Calv = cortical alveolar stage oocyte, EVit = early vitellogenic stage oocyte, Fo = follicle cells, GV = germinal vesicle, LVit = late vitellogenic stage oocyte, MVit = middle vitellogenic stage oocyte, OD = oil droplet, YG = yolk granule, ZR = zona radiata. Scale bar 100  $\mu\text{m}$ .

Figure 1-7. Maturing oocyte (stage IVa) in a *Z. flavescens* female sampled at 9 am. The germinal vesicle (GV) is in the process of migrating to the periphery of the ooplasm. Fo = follicle, O = oil droplets coalescing, Y = yolk granules coalescing. Scale bar 100  $\mu\text{m}$ .

about 3  $\mu\text{m}$  (Table 1-2). The GV:cytoplasm ratio was approximately 0.36 (Table 1-2).

### IIIc) *Late vitellogenesis*

*Size range: 202-318 $\mu\text{m}$  (mean diameter = 271.1  $\pm$  26.8  $\mu\text{m}$ )*

Oocyte diameter and the number and size of yolk granules and oil droplets increased further in the late vitellogenic stage (Figure 1-6). Yolk granules increased in size up to approximately 13  $\mu\text{m}$  in diameter; large oil globules of all sizes were observed, up to 24.5  $\mu\text{m}$  in diameter (Table 1-2). The cell cytoplasm was completely filled with both oil and yolk granules, and no 'empty' cytoplasm was visible, as it was in earlier stages. The ZR widened to about 9.7  $\mu\text{m}$  (Table 1-2) and stained intensely with eosin. The GV:cytoplasm ratio was approximately 0.3 (Table 1-2).

## IV. Mature stage

To ensure neutral buoyancy in sea water and thus proper dispersal upon spawning, oocytes of *Z. flavescens* and other pelagic spawning species undergo the process of final maturation concomitantly with hydration (Wallace and Selman, 1981; Finn *et al.*, 1999). In the present study, maturing stage IVa oocytes were observed in fish sampled at least 3 hours, and up to 12 hours, prior to spawning (Table 1-3). Both fully hydrated and ovulated oocytes (*i.e.*, oocytes in stage IVb), having already completed maturation, were observed in fish sampled within 2 hours of spawning (Table 1-3). In other words, within 2 hours before spawning, all stage IV oocytes in the ovaries had progressed to stage IVb. Mature stage oocytes (stage IVa or IVb) were observed in 134 of 188 total ovaries (71% of those examined).

### IVa) *Maturing stage*

*Size range: 340-596 $\mu\text{m}$  (mean diameter = 452  $\pm$  61.0  $\mu\text{m}$ )*

In this stage, coalescence of yolk granules accompanied the dissolution of the nucleus,

Table 1-3. Patterns of oocyte maturation in *Z. flavescens* sampled over daylight hours. All collections of fish were taken in 2005 except the 1730 collection which was taken in 2006. Spawning occurred within 1/2 hour of sunset, which varied by time of year. The earliest spawning observed (during winter months) was after 1730 hours.

<i>Time (hrs)</i>	<i>Fish (N)</i>	<i>No. ovaries with oocytes in stages I-III</i>	<i>No. ovaries with oocytes in stage IVa</i>	<i>No. ovaries with oocytes in stage IVb</i>
0900	3	3	2	0
1000	2	2	2	0
1100	1	1	1	0
1200	3	3	2	0
1300	4	4	4	0
1400	13	13	5	3
1500	3	3	2	1
1600	1	1	0	1
1700	2	2	0	2
1730	6	6	0	6

or Germinal Vesicle Break Down (GVBD, as defined by Goetz, 1983), and the migration of the GV to the periphery of the cytoplasm. Both processes are commonly used as indicators of oocyte maturation (DeVlaming, 1983) (Figure 1-7). Concurrently, the maturing stage oocyte began the process of hydration, as noted by its marked increase in diameter when compared to stage III oocytes (Table 1-1). Maturing oocytes were un-ovulated, and still surrounded by the follicle.

#### *IVb) Hydrated stage*

*Size range: 500-900 $\mu$ m (mean diameter = 734.5  $\pm$  63.2  $\mu$ m)*

Because 'wrinkling' of the oocyte membrane due to the dehydrating histological preparation of the tissue created a distorted appearance of stage IVb oocytes (Figure 1-8), an accurate description of oocyte morphology from histological samples at this stage proved impossible. However, a distinction between fully hydrated (IVb) and maturing (IVa) oocytes was possible due to the obvious differences in shape of the two stages (Figures 1-7 and 1-8). Some fully hydrated oocytes were observed in the ovarian lumen, having been ovulated prior to sampling of the female examined.

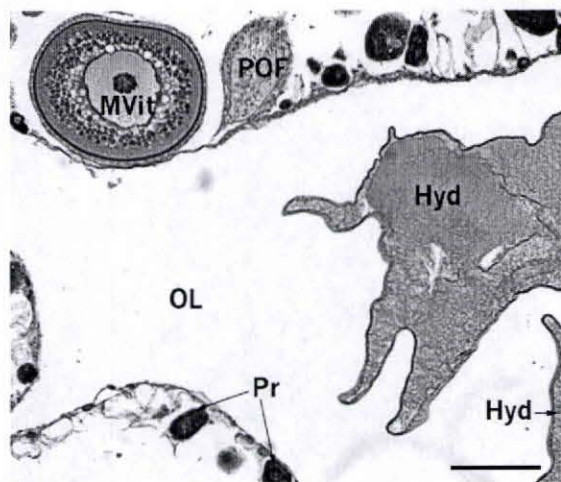


Figure 1-8. Hydrated oocytes (stage IVb) in the ovarian lumen of a *Z. flavescens* individual sampled 2 hours prior to sunset. Fully hydrated oocytes have been ovulated and remain within ovarian lumen, awaiting spawning. Hyd = hydrated oocytes, MVit = middle vitellogenic stage oocyte, OL = ovarian lumen, POF = post ovulatory follicle. Pr = primary oocytes. Scale bar 100  $\mu$ m.

In addition to the documentation of oocyte maturation by stage, histological investigation permitted the observation of ovarian composition of oocyte populations for all histologically processed ovaries. All healthy ovaries from adult females contained oocytes in the primary growth stages (Ia and Ib), cortical alveolar stage (II), and all three vitellogenic stages (IIIa, IIIb and IIIc). In addition, 134 out of 188 total ovaries examined also contained stage IV oocytes. Ovaries from two females appeared unhealthy and were omitted from the analysis.

The intentional sampling of females at different times of day, starting at 0900 hours and continuing until 1730 hours, illuminated the daily cycle of egg production in this species. As early as 0900 hrs, maturing stage (IVa) oocytes were visible within the ovary, together with oocytes of all previous stages (Table 1-3). Throughout the day, maturing oocytes became enlarged due to hydration (Figure 1-7), and at about 2



hours prior to sunset (*i.e.*, between 1400 and 1500 hrs, depending on the time of year) hydrated stage oocytes (IVb) were observed within the ovaries (Table 1-3). Ovaries from individuals with no stage IV oocytes were also observed at all times of the day (Table 1-3), indicating that egg release occurs around sunset, but that not all females release eggs every day.

Additionally, some atretic follicles were observed within individual ovaries, both of unspawned maturing stage and vitellogenic stage oocytes, although they did not occur frequently enough to warrant any assumptions concerning their role in the regression of the ovaries after spawning (DeVlaming, 1983; Santiago and Sanz, 1992; Tyler and Sumpter, 1996). Atretic follicles were easily distinguished from POFs by their lack of a follicular lumen and by the presence of spherical oil droplets.

#### ***Documentation of post-ovulatory follicle (POF) degeneration***

Following the methods of Van den Hurk and Peute (1979) and Hunter *et al.* (1985), this section documents the degeneration of POFs over a 48-hour period using captive fish sampled at 6-hour time intervals. POFs were no longer visible in

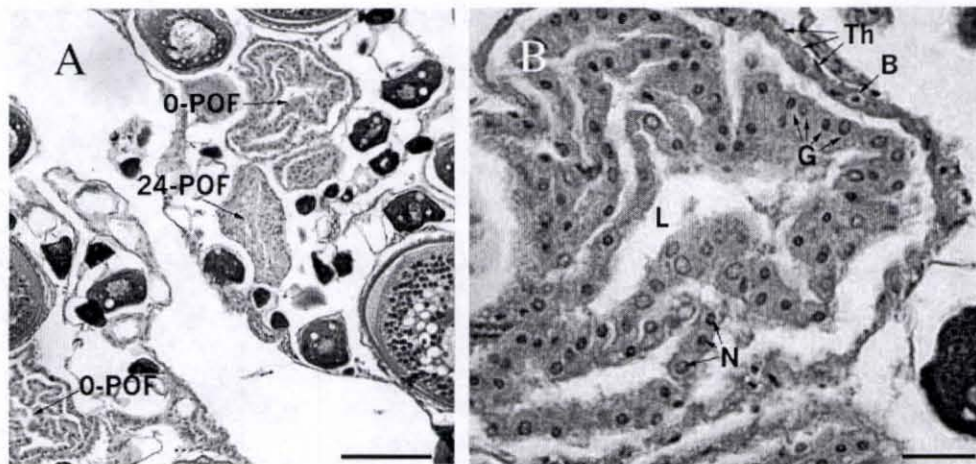


Figure 1-9. (A) Zero- and 24-hour POFs in a *Z. flavescens* ovary and (B) the same zero-hour POF at higher magnification. B = blood capillary G = granulosa cells, L = lumen, N = granulosa cell nuclei, Th = theca cell. Scale bars (A) 100  $\mu$ m and (B) 20  $\mu$ m.

fish euthanized at 42-hr and 48-hr time points post egg-release, indicating that in *Z. flavescens*, the resorption of follicular cells and associated structures and molecules was completed between 36 and 42 hours after ovulation.

**Time: 0-hr**

In ovarian tissue removed from a female directly following egg release, the empty follicle cell layers, composed of theca and granulosa cells, formed many loose folds, which together formed the POF (Figure 1-9B). Theca cells were tightly associated with granulosa cells on the outer 'rim' of the POF (Figure 1-9) and were harder to distinguish toward the interior region. The granulosa cells were cuboidal at this early stage, with cell wall boundaries defined and clearly evident. Most granulosa cell nuclei were round and distinct, staining uniformly (Figure 1-9B).

The most obvious characteristic of this stage was the continuous chain of granulosa cells which could be followed easily by eye. The follicular lumen between granulosa cell folds was at its widest relative to older POFs (Figure 1-9A). Theca and granulosa cells were closely associated with blood capillaries in 0-hr POFs and at later stages of POF

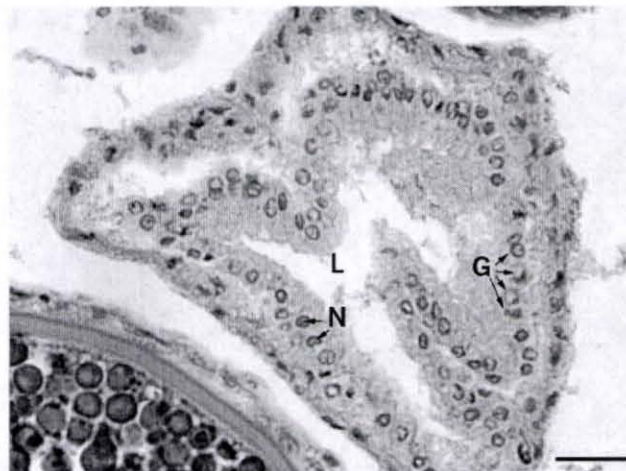


Figure 1-10. Six-hour POF in a *Z. flavescens* ovary. G = granulosa cells, L = lumen, N = granulosa cell nuclei. Scale bar 20  $\mu$ m.



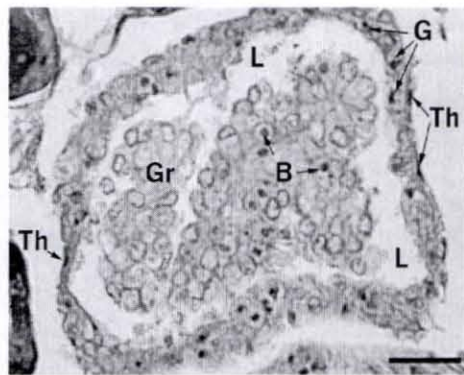


Figure 1-11. Twelve-hour POF in a *Z. flavescens* ovary. B = blood capillary, G = granulosa cells, Gr = atrophying granulosa cell mass, L = lumen, Th = theca cell. Scale bar 20  $\mu$ m.

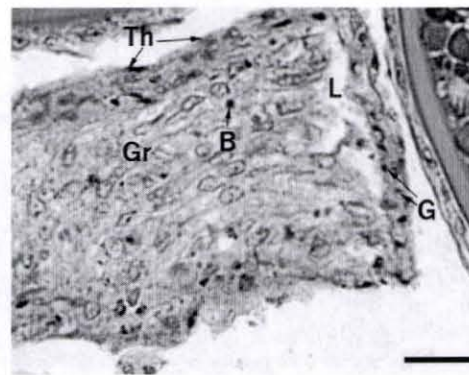


Figure 1-12. Eighteen-hour POF in a *Z. flavescens* ovary. B = blood capillary, G = granulosa cells, Gr = atrophying granulosa cell mass, L = lumen, Th = theca cell. Scale bar 20  $\mu$ m.

degeneration. All granulosa cells stained approximately the same intensity with eosin at this 0-hr time point.

#### Time: 6-hr

In comparison to 0-hr POFs, the lumen of 6-hr POFs was smaller in area and slightly less defined by granulosa cell walls that sometimes appeared 'blurred' (Figure 1-10), suggesting the beginning of cellular degradation. An observer was able to follow the line of granulosa cells as on a time 0-hr POF, but the continuous formation was less distinct in 6-hr POFs, and included gaps where cellular structures appeared less organized. Granulosa cell nuclei were still circular, but stained lighter in intensity than in time 0-hr POFs. Most granulosa cells remained cuboidal; very few were elongated.

#### Time: 12-hr

The lumen of 12-hr POFs shrank in width and remained only as a narrow space between an inner region of deteriorating granulosa cells and an outer portion of relatively intact granulosa and theca cells (Figure 1-11). The walls of granulosa cells in the inner region of the POF began to disappear, leaving behind an indistinguishable mass of degrading

cellular components. Inner granulosa cell remnants stained less intensely than outer granulosa cells, and appeared to be undergoing disintegration at a faster rate than granulosa cells on the outer portion of the POF, since outermost granulosa cells still retained dark nuclei and more often maintained a cuboidal shape. Theca cells did not exhibit signs of disintegration or atrophy; they appeared to retain both size and shape and maintained distinct cellular boundaries (Figure 1-11).

**Time: 18-hr**

The POF lumen was further compressed, and in some cases, was entirely absent from 18-hr POFs. When present, the lumen was very thin and appeared only between the inner cell mass and the outer cellular boundary (Figure 1-12). The inner cell mass still stained with less intensity than outer cells, and was more disorganized in appearance than in earlier POFs. In the interior of the 18-hr POF, granulosa cells no longer maintained a cuboid shape; instead, they appeared to have lost internal cellular integrity, and cells housed only lightly stained and elongated nuclei, if any. Disorganized granular material of cell fragments filled the entire center region of the POF. Theca cells appeared unchanged on the outer rim of the POF. A few remaining intact granulosa cells could also be seen on the outer rim of the POF, associated with the theca cells.

Table 1-4. The size ranges of POFs at 2 time points during degeneration in *Z. flavescens* ovaries. Time points were chosen so as to be able to assess differences in size (and therefore 'age') of POFs observed in ovaries sampled at approximately 1.5-2 hours prior to spawning. All measurements are given in microns. No POFs were observed 48 hours post spawning.

<i>Time post egg release</i>	<i>Size range (mean <math>\pm</math> std. dev.)</i>	<i>No. POFs measured</i>
POF 0 hr	$6.96 \times 10^3 - 51.60 \times 10^3$ ( $18.24 \times 10^3 \pm 8.36 \times 10^3$ )	99
POF 24 hr	$2.51 \times 10^3 - 13.33 \times 10^3$ ( $6.63 \times 10^3 \pm 2.14 \times 10^3$ )	99



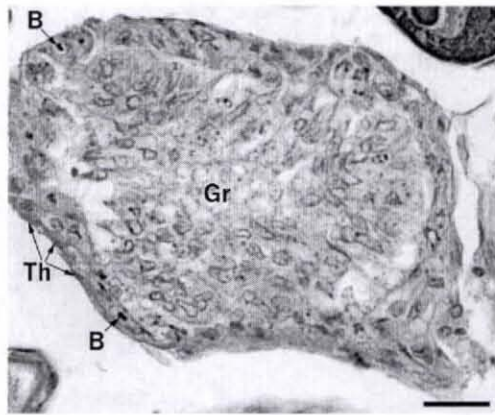


Figure 1-13. Twenty-four-hour POF in a *Z. flavescens* ovary. B = blood capillary, Gr = atrophying granulosa cell mass, Th = theca cell. Scale bar 20  $\mu$ m.

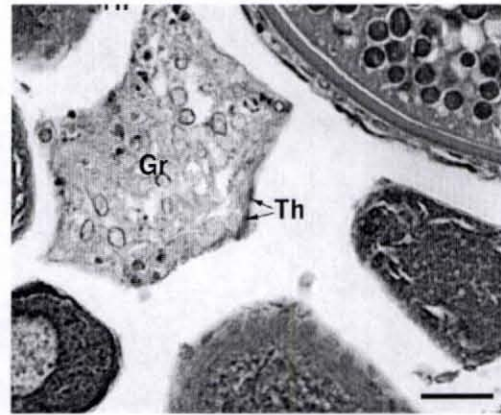


Figure 1-14. Thirty-hour POF in a *Z. flavescens* ovary. Gr = atrophying granulosa cell mass, Th = theca cell. Scale bar 20  $\mu$ m.

#### Time: 24-hr

The former lumen of 24-hr POFs was completely absent, although a distinction between the outer cell layer and the inner cell mass could be made due to the more intense staining of the outer rim of the POF (Figure 1-13). Outer theca cells remained intact and stained similarly to those in 0-hr POFs and pre-ovulatory follicles.

The central area of the POF was the most disorganized region: granulosa cell walls were no longer distinguishable except in some outer portions of the POF. The size of a 24-hr POF tended to be about half that of a 0-hr POF (Table 1-4). A 24-hr POF was easily distinguished from a 0-hr POF within the same ovary at 10x magnification (Figure 1-9) due to obvious differences in size and cellular composition, as well as presence (in 0-hr POFs) and absence (in 24-hr POFs) of the follicle lumen.

#### Time: 30-hr

The overall size of 30-hr POFs was smaller than 24-hr POFs. Inner granulosa cells were increasingly disorganized, with only a few retaining cell walls and visible nuclei (Figure 1-14). Inner cell remnants stained with very low intensity. Theca cells on the periphery



Figure 1-15. Thirty-six-hour POF in a *Z. flavescens* ovary. Gr = atrophying granulosa cell mass, Th = theca cell. Scale bar 20  $\mu$ m.

of the POF continued to stain with previous intensity and appeared unchanged during the degeneration process.

#### **Time: 36-hr**

Thirty-six-hour old POFs tended to be smaller, but otherwise were very similar in appearance to 30-hr POFs (Figure 1-15).

#### **Time: 42-hr and 48-hr**

No POFs were visible in ovaries sampled 42 hours after ovulation.

Using these documented descriptions from each time point, in addition to the difference in mean size of POFs at 0 and 24 hours (Table 1-4), it was possible to distinguish between empty follicles that were remnants of eggs just released (0-hr POFs) and follicles that were residual from the previous day's spawn (24-hr POFs) as illustrated in Figure 1-9. This distinction provided histological evidence for or against previous-day spawning in every female examined.

## DISCUSSION

The establishment of clearly defined stages of oocyte maturation and POF degeneration within the ovary of *Z. flavescens* can now be used to investigate temporal changes in oocyte population composition within and among individuals, as will be demonstrated in Chapter 2 of this thesis. Based on this histological investigation of oocyte populations within individual ovaries, further conclusions concerning the pattern of egg maturation in *Z. flavescens* were also suggested.

### *Oocyte staging and maturation patterns*

Development of oocytes in *Z. flavescens* is classified as asynchronous, based on the simultaneous presence of oocytes in several stages of development within the ovary (Figures 1-4 through 1-8). In ovaries of fish with asynchronous oocyte development, recruitment of oocytes from an earlier stage to the next stage of development occurs as a continuous process during the breeding season, with different oocytes at different stages (and therefore different sizes) together concurrently within an ovary (Scott, 1987; Selman and Wallace, 1989). By definition, fish with asynchronous oocyte populations in the ovary are multiple spawners (Scott, 1987; Tyler and Sumpter, 1996), ovulating several batches of the most mature stage oocyte over the spawning season, while earlier stage oocytes remain within the ovary to mature and differentiate and are released at later spawning events. Fecundity of this multiple-spawning fish is therefore indeterminate (as defined by Hunter and Macewicz (1985)), since the number of eggs produced by an individual over a season cannot be known without an understanding of frequency of spawning or the number of eggs released per spawn.

### *Time of spawning*

Observations of pre-sunset spawning behavior in Kona by Walsh (1984) and similar behaviors at dusk by captive fish from the same location (C.W. Laidley, pers.

comm.) both suggest pre-sunset spawning in this species. Spawning of males and females in this population has been observed within a distinct window of time during the one hour prior to sunset, with no instance of *Z. flavescens* spawning reported later than 10 minutes post-sunset (Walsh, 1984).

In this study, histological evidence from adult female *Z. flavescens* sampled at different times of day offers further support for early evening egg release. Full progression of oocytes through the final stage (stage IV) occurs throughout an ~ 8-hour period leading up to pre-sunset spawning (Table 1-3). Maturing (stage IVa) oocytes were observed within ovaries as early as 0900 hrs, and the maturation and hydration processes continued throughout the day (Table 1-3 and Figure 1-7). Ovulated eggs were observed in the lumen of the ovary beginning about 2 hours prior to sunset (Table 1-3 and Figure 1-8). Similarly, the closely related brown surgeonfish, *Acanthurus nigrofuscus*, spawns daily during its reproductive season, with egg maturation and hydration occurring over a similar time span (Fishelson *et al.*, 1987). Evening spawning has been noted in several species of coral reef fishes, including other acanthurids (Randall, 1961a and b; Lobel, 1989; Craig, 1998). Reasons postulated for evening spawning include the decreased likelihood of predation on newly released eggs by diurnal planktivores (Lobel, 1978; May *et al.*, 1979; DeMartini and Fountain, 1981), as well as decreased threat of predation on the adult spawners (Johannes, 1978).

### ***Frequency of spawning***

With the exception of 2 individuals with apparently unhealthy gonads, all adult females (N=188) from year-round field collections exhibited similar oocyte composition, with oocytes in stages I, II, and III present in every ovary examined. In addition, mature stage (IVa and b) oocytes were also observed in a fraction of all females sampled (Table 1-3). The presence of hydrated stage (IVb) oocytes within an ovary sampled prior to sunset indicated that an individual would have spawned that evening had it not been

caught; conversely, the absence of hydrated stage oocytes indicated that that female would not have spawned that evening. However, absence of stage IV oocytes did not signify the female's potential to spawn on ensuing days, or whether she had spawned previously. Notably, the presence of 24-hour-old POFs (*i.e.*, POFs similar in appearance to those examined from captive fish, 24 hours post egg release) did indicate day-prior spawning in certain individuals.

Previous studies have incorporated the presence and 'age' of POFs into estimations of spawning frequency of populations of indeterminate spawning fishes (*e.g.*, Hunter and Goldberg, 1980; Hunter *et al.*, 1985; Santiago and Sanz, 1992; Sanz *et al.*, 1992; Macchi *et al.*, 1999; Stequert *et al.*, 2003; Jackson *et al.*, 2006). While most of these studies used very large sample sizes to approximate the number of days between spawning for females within the targeted population, the current study was limited in its ability to sample suitable numbers of fish to make such an estimate of population-wide spawning frequency. Instead, the determination that POFs remain in the ovary of this tropical species for up to 42 hours, and the identification of POFs from the previous day's spawn, contribute to our understanding of the prevalence of consecutive-day spawning for this population of *Z. flavescens*. Clearly, a close approximation to the actual frequency of spawning for individual females cannot be determined by histological investigation alone. A different approach to estimating spawning frequency in the sampled population of *Z. flavescens* is addressed in Chapter 3.

## **CHAPTER 2.**

### **An investigation of *Zebrasoma flavescens* (yellow tang) spawning seasonality including lunar periodicity**

#### **ABSTRACT**

To answer questions concerning annual spawning seasonality and to establish the presence or absence of a lunar cycle of spawning, reproduction of *Zebrasoma flavescens* (yellow tang), a popular aquarium species harvested from the Kona coast of the Big Island of Hawai'i, was investigated. The results of this study demonstrated that female *Z. flavescens* produce mature oocytes year round. However, increases in female gonadosomatic index (GSI), batch fecundity, and incidence of females spawning for at least two consecutive days were observed in spring and summer months, indicating a protracted spawning season with greater reproductive output for the population during March-July. Female condition (as defined by Froese (2006)) did not change significantly throughout the year. A lunar cycle of spawning, with an observed increase in batch fecundity and female GSI in the 4 days prior to and the day of the full moon, was observed in May through July (batch fecundity: periodic regression,  $F = 32.5$ ,  $df = 2$ , 219,  $p < 0.001$ ; GSI: periodic regression,  $F = 38.8$ ,  $df = 2$ , 225,  $p < 0.001$ ). In addition, the proportion of oocytes in the late vitellogenic stage within yellow tang ovaries sampled in the summer months also exhibited significant lunar periodicity (periodic regression,  $F = 9.5$ ,  $df = 2$ , 52,  $p < 0.001$ ). These results provide the basis for estimating egg production for yellow tang populations in Kona, Hawai'i and can support the development of management measures in this and other locations under aquarium fishing pressures.

#### **INTRODUCTION**

Successful reproduction depends upon a specific series of physiological and behavioral processes, including oocyte (egg-precursor cell) production, spawning,

fertilization, and gamete maturation and survival. Because the first step is female-specific, research related to the initial requirements for reproduction in this study focuses on the female sex. The initial processes in *Zebrasoma flavescens* reproduction - oocyte production and maturation - were investigated and described in detail in Chapter 1. This chapter will explore temporal trends in spawning for females of this species, including an investigation into annual seasonality, if any, and the presence or absence of a lunar cycle of spawning.

It is not unusual for marine tropical reef fish species to exhibit a more protracted spawning season compared to temperate fish species (Lowe-McConnell, 1979); the length of the reproductive season has been shown to increase with decreasing latitude of populations (Conover, 1992). Instead of sharp delineations between 'reproductive' and 'non-reproductive' periods, many tropical species exhibit periods of intermediate spawning production at one or both ends of the season (Colin and Clavijo, 1988; Figuerola *et al.*, 1998; Kritzer, 2004). Alternatively, as exhibited by some species of Hawai'ian reef fishes, reproduction within a tropical population may continue year-round, but with periods of greater reproductive effort concentrated in specific months (Ross, 1982; Walsh, 1987; Lobel, 1989; Privitera, 2002).

The few specific studies of reproductive seasonality within the surgeonfish family Acanthuridae have shown significant differences in spawning patterns among species. *Acanthurus nigrofuscus*, which is found on coral reefs all over the world and in large numbers on the Kona Coast, exhibits a clear demarcation between on- and off-season spawning months in the Red Sea (Fishelson *et al.*, 1987). Ovaries of *A. nigrofuscus* females undergo large-scale atresia of oocytes following the breeding season, and no mature oocytes are produced in off-season months (Fishelson *et al.*, 1987). Randall (1961a) found *Acanthurus triostegus* in Hawai'i to have a distinct spawning season, based on gonadosomatic index (GSI) of males and females sampled over one year. In



contrast, *A. triostegus*, *Acanthurus guttatus*, and *Acanthurus lineatus* were found to spawn year-round in American Samoa (Craig, 1998), although with periods of more intense reproductive effort in the austral summer months.

It is likely that *Z. flavescens* in Hawai'i also participate in some level of spawning throughout the year, based on year-round observations of spawning behavior (Lobel, 1989) and the continuous presence of new recruits (though often at low levels) on the reef in Kona populations (Walsh, 1987; J.T. Claisse and M.E. Bushnell, unpub. data). Captive fish have also been found to spawn during all months of the year (C.W. Laidley, unpublished data). The *Z. flavescens* spawning season has been previously investigated in Kona (Lobel, 1989) and the island of Oahu, Hawai'i (C.W. Laidley, pers. comm.). Both studies indicated a peak in reproductive effort, defined here as increasing GSI values for males and females, beginning in late March and continuing through August. However, Lobel (1989) sampled in areas of the reef probably frequented by juveniles, resulting in samples of small fish with low GSI values on which his spawning seasonality conclusions were based. Therefore, a more thorough investigation of yellow tang spawning seasonality was pursued for the Kona population in the current study.

Lunar periodicity among repetitive spawning species has also been established for many marine tropical fish species (e.g., Lobel, 1978; May *et al.*, 1979; Robertson *et al.*, 1990; Mizushima *et al.*, 2000; Soyano *et al.*, 2003; Takemura *et al.*, 2004; Vagelli and Volpedo, 2004), and has been suggested for some acanthurids (Randall, 1961a and b; Thresher, 1984; Colin and Clavijo, 1988). Randall (1961a and b) observed a greater number of ripe females around the full moon among populations located on Oahu (*A. triostegus*) and the Society Islands (*Zebrasoma scopus*), whereas Colin and Clavijo (1988) observed peak spawning of *Acanthurus coeruleus* between 3 and 8 days after the full moon in a Puerto Rico population. Preliminary sampling of a Kona population of *Z. flavescens* in 2005 indicated an increase in both the fraction of females producing eggs



and the number of eggs released by individuals around the full moon (J.T. Claisse and M.E. Bushnell, unpublished data). In addition, fish held in captivity were observed to spawn more eggs and more frequently during the full moon quarter than at other times of the month (C.W. Laidley, unpublished data). Therefore, the primary objectives of this project were: (1) to establish the period of greatest reproductive output (*i.e.*, annual seasonality) for a yellow tang population located on the Kona coast of Hawai'i, by collecting adult females for one calendar year and analyzing batch fecundity and GSI for possible annual trends, and (2) to verify the presence or absence of a lunar cycle of spawning for *Z. flavescens* using the same measurements.

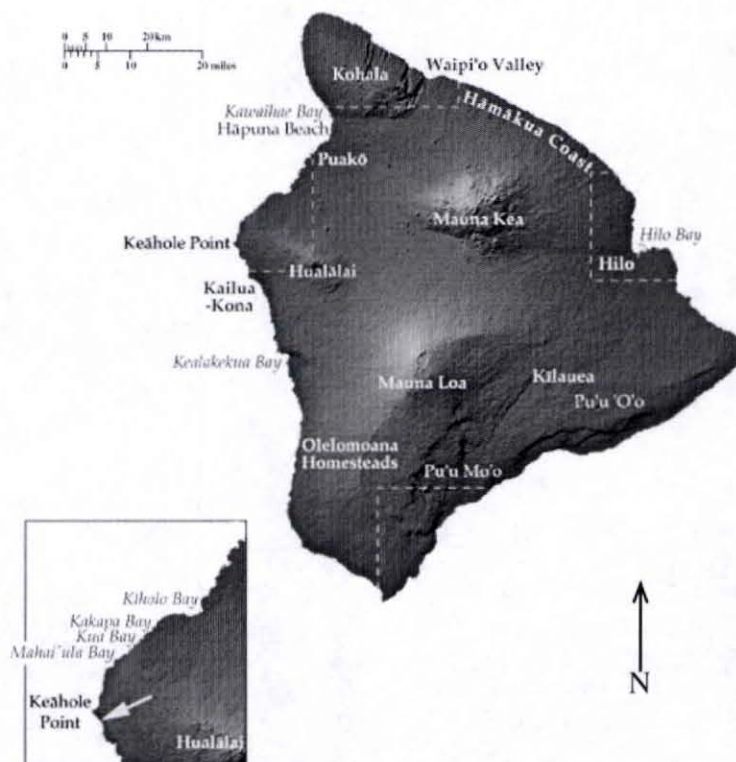


Figure 2-1. Map of Hawai'i Island and inset of part of the Kona Coast. Arrow indicates sampling area (N19° 42', W156° 03'), approximately 3 km in length. Image courtesy of Hawai'i Mapping Research Group, University of Hawai'i.

## MATERIALS AND METHODS

### *Sampling*

The area sampled was located on the western coast of Hawai'i Island (N19° 42', W156° 03') and is illustrated in Figure 2-1. Intensive sampling (*i.e.*, approximately every 4 days) was performed during the period of the year that had been hypothesized to be the peak of the spawning season, based on previous studies in Kona (Walsh, 1987; Lobel, 1989), as well as earlier research on Oahu (C.W. Laidley, pers. comm.).

### *Monthly collections*

Sampling was performed once per month for one calendar year, from May 1, 2006

Table 2-1. Sample sizes of adult *Z. flavescens* Group M and Group S fish. N(month) is the number of fish caught in the corresponding monthly collection. N (day) is the number of fish caught on the corresponding day of the summer sampling period. N(histo) is the number of fish examined histologically for presence of POFs and for vitellogenic oocyte-size-frequency distributions in Figure 3-7. Cells shaded in gray denote the same sample of fish used in both groups.

<i>Month</i>	<i>N (month)</i>	<i>Day of Sampling Period (summer 2006)</i>	<i>N (day)</i>	<i>N (histo)</i>
<i>Group M fish</i>		<i>Group S fish</i>		
May 2006	12	0	6	
Jun 2006	16	4	12	7
Jul 2006	7	6	17	
Aug 2006	-	9	15	6
Sep 2006	17	13	11	6
Oct 2006	9	18	11	6
Nov 2006	14	24	14	
Dec 2006	17	28	11	6
Jan 2007	19	31	10	
Feb 2007	21	33	16	6
Mar 2007	18	35	16	6
Apr 2007	19	38	14	
		42	11	6
		48	11	6
		51	16	
		58	16	6
		61	11	6
		63	7	5

through April 3, 2007, excepting August 2006. On either the day before, the day after, or the day of the full moon, between 7 and 21 female fish were sampled using 3-pronged spears (Table 2-1). Two divers using SCUBA speared fish until either air supplies became low (after approximately 45-55 min) or until at least 20 female fish were obtained, whichever came first. Fish were collected in 8-10 m of water inshore from the reef crest, at a location previously determined by J.T. Claisse (unpublished data) to be adult daytime habitat. All monthly sampling dives were conducted within 2 hours of sunset in order to minimize effects of egg hydration on ovary weight, since a positive relationship between time of day and ovary weight had been noted in preliminary collections (Figure 2-2).

No collection was made in August of 2006 due to weather complications. Each monthly collection of female fish was obtained on one sampling day except the December 2006 collection, which encompassed two days. On December 4 (the full moon), 2006, only 8 females were obtained, so sampling was also conducted on the following day, December 5, on which 9 females were collected. There was no statistical difference in

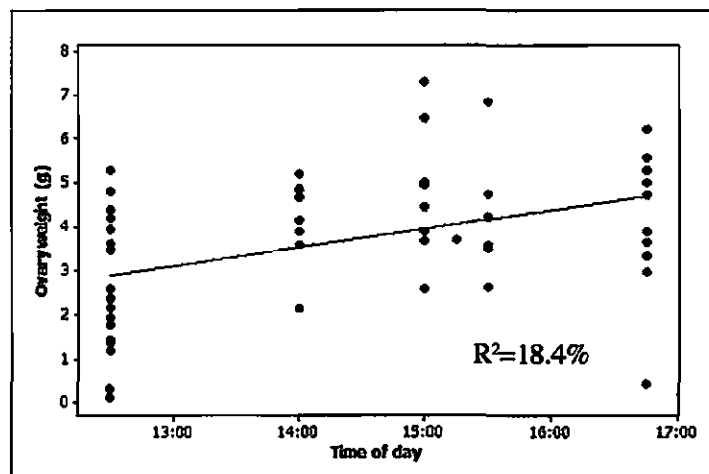


Figure 2-2. Ovary weight of *Z. flavescens* females sampled in preliminary collections in 2005 from the Kona Coast. A significant relationship between ovary weight and time of day was established (linear regression equation: Ovary weight = - 2.46 + 10.3 Time of day,  $p < 0.001$ ) due to the progressive hydration of eggs in the ovary over the course of daylight hours.

average fecundity (t-test,  $p < 0.61$ ) or GSI (t-test,  $p < 0.84$ ) between females collected on the two sampling days, so for the month of December, GSI and fecundity values for the two days were pooled. Fish sampled at the monthly peak were considered to be Group M fish (M=monthly), and are referred to as such in the remainder of the text.

#### *Summer collections*

For a 65-day period lasting from May 8 to July 10, 2006, between 7 and 17 female fish were sampled approximately every 4 days for a total of 18 collections (Table 2-1). Sampling protocols were identical to those used in monthly sampling. Fish collected during the summer months are considered Group S fish (S=summer) for the remainder of the text. Data from this 65-day intensive sampling period were applied to the remainder of the year-round sampling effort in order to maximize sampling of reproductively active adults.

#### *Fish processing*

Fish were placed on ice after spearing, and gonad dissections commenced within 12 hours. Each fish was weighed, sexed, and measured for total length (TL) and standard length (SL). All gonads were dissected, weighed, and placed either on ice for batch fecundity counts or into Dietrich's fixative for histological processing.

#### *Histological processing*

(See methods section of Chapter 1 for detailed descriptions of histological embedding and sectioning protocols.) For frequency distributions of vitellogenic stage oocytes (stage III), all vitellogenic oocytes from a cross-section of an ovary were measured using QCapturePro software, and size-frequency distributions based on the diameter of oocytes in each of the three vitellogenic stages, as defined in Chapter 1, were calculated.

#### *Ovary sampling*

Pilot histological data (presented in Table 2-2) suggested no difference in oocyte

population composition between the anterior, middle, or posterior portions of yellow tang ovaries, nor between left and right lobes of ovaries taken from the same individual. Histological slides of cross-sectional diameters were created from the anterior, middle and posterior area of each of 3 ovaries (both lobes). The area of each cross-sectional diameter taken up by vitellogenic (stage III) oocytes was calculated using ImageJ software (open-source), and a t-test was performed on data from the left and right lobe of each of the 3 ovaries. No significant difference between left and right lobes of the ovaries from the three individuals was found (two-sample t-tests,  $p < 0.24$ ,  $p < 0.13$ ,  $p < 0.17$ ), and the data presented in Table 2-2 indicate minimal differences in oocyte composition between anterior, middle and posterior portions of the ovaries.

Nevertheless, every effort was made to sample consistently from the same area of each ovary. Subsamples were always taken from the middle portion of the ovarian lobe, just anterior of the joining of the two lobes, and each subsample weighed at least as much as 10% of the total weight of the sampled lobe.

Table 2-2. Ovary composition of 3 *Z. flavescens* females (individuals are distinguished by letters A, B and C) based on histological data. The entire cross-sectional diameter of each ovary was sampled from three consistent areas of the ovary: in the anterior, middle, or posterior portion. The fraction of the total cross-sectional area of the ovary taken up by all stages of vitellogenic oocytes (stage III) is given. The last column presents the results of a two-tailed t-test comparing the composition of the left and right lobes of each individual's ovary. No significant difference between composition of the two lobes was found for any of the 3 fish.

<i>Ovary</i>	<i>Anterior</i>	<i>Middle</i>	<i>Posterior</i>	<i>T-test</i>
A left	0.211	0.269	0.288	$p < 0.24$
A right	0.276	0.282	0.400	
B left	0.723	0.662	0.650	$p < 0.13$
B right	0.769	0.796	0.691	
C left	0.608	0.551	0.639	$p < 0.17$
C right	0.634	0.639	0.664	

### *Fecundity measurements*

Batch fecundity, defined here as the number of eggs released by a female during a single spawning event, was considered for the purposes of this study to be equal to the number of hydrated oocytes and ovulated eggs within an ovary sampled prior to spawning (See Chapter 1 for definitions and photographs of hydrated oocytes). Any hydrated oocytes remaining within the follicle at the time of dissection were assumed to be close to ovulation, as females were intentionally sampled within 1.5-2 hours before spawning. (For simplicity, the term 'eggs' is used for the rest of this thesis to refer to all hydrated oocytes and ovulated eggs counted within an ovary.)

For batch fecundity measurements of fish ovaries containing approximately 3000 eggs or less, both the left and right lobes of the ovary were dissected out and weighed. One lobe was randomly assigned to use for batch fecundity counts and the other was preserved for histology (See Chapter 1). For batch fecundity counts, all eggs were counted from a weighed subsample of the chosen lobe. When an ovary lobe weighed less than 1.0 g, all eggs from the entire left or right lobe of the ovary were counted.

The number of eggs counted from the subsample was then used in the following equation to estimate batch fecundity of each fish:

$$BF = N \times \frac{L_w + R_w}{S_w} \quad (\text{EQUATION 2-1})$$

where BF = batch fecundity, N = number of eggs counted in subsample,  $L_w$  = weight of left ovary in grams,  $R_w$  = weight of right ovary in grams, and  $S_w$  = weight of subsample in grams.

The previously described method of subsampling was sufficient if the ovary contained less than ~3000 eggs. If more than ~3000 eggs were present, spillage from the genital opening was likely to occur during dissection, and determination of the lobe from which the eggs originated became impossible. In these cases, the following method was used.

For the highest fecundity ovaries (those with 4000-28,000 eggs), eggs were extracted

from within the fresh ovary by physically massaging both lobes into a Petri dish and spilling the eggs out of the ovary via the lumen. Eggs and ovarian tissue were then weighed separately, and a subsample of the eggs was weighed and counted. The number of eggs counted from the egg subsample was then used in the following equation to estimate batch fecundity of each fish:

$$BF = N \times \frac{E_w}{S_w} \quad (\text{EQUATION 2-2})$$

where BF = batch fecundity, N = number of eggs counted in egg subsample,  $E_w$  = weight of all eggs within ovary in grams, and  $S_w$  = weight of egg subsample in grams.

#### *Condition factor*

Condition factor was calculated using the following equation (Froese, 2006):

$$K = \frac{W - G_w}{SL^3} \times 10^6 \quad (\text{EQUATION 2-3})$$

where K = Fulton's condition factor, W = total weight of the fish in grams,  $G_w$  = total weight of the gonad in grams, SL = standard length in millimeters, and C = species-specific constant.

#### *Tidal data compilation*

Tidal data for the sampling location were obtained from the National Oceanic and Atmospheric Administration website (NOAA.tidesandcurrents.com) and are presented in Table 2-3. Times for high and low tides collected at the tidal station at Kawaihae, Hawai'i, station ID number 1617433, were adjusted for the Kailua-Kona harbor (N19° 39', W156° 00'), the nearest available location with a time conversion.

#### *Data analysis*

Statistical analyses were performed using Minitab 14 software. Periodic

regressions used the following equations (Cryer, 1986; deBruyn and Meeuwig, 2001):

$$Y = b_0 + b_1(\cos\theta) + b_2(\sin\theta) \quad (\text{EQUATION 2-4})$$

where  $Y$  is the dependent variable (batch fecundity or GSI),  $b_0$  is the mean level of  $Y$ ,  $b_1$  and  $b_2$  are model coefficients which together define the phase shift and amplitude of the sine wave, and

$$\theta = \frac{2\pi t_n}{f} \quad (\text{EQUATION 2-5})$$

where  $t_n$  is the original time variable (day of sample) and  $f$  is the frequency of the expected pattern. For the lunar cycle, the frequency of recurrence of the pattern was computed as 28 days.

Table 2-3. Tidal records for Kona, Hawai'i during the year sampled. Tidal amplitude is given in meters relative to mean sea level. HST = Hawaii Standard Time, HHW = higher high water tide, LHW = lower high water tide. \*Sampling was not conducted in May 2007, although tidal information is included in the table to better assess annual tidal patterns.

<i>Month</i>	<i>Full moon date</i>	<i>High tide (m)</i>	<i>High tide HST</i>	<i>Low tide (m)</i>	<i>Low tide HST</i>	<i>Sunset HST</i>	<i>Tidal description</i>
May 06	12	.401	15:44	-.199	22:59	19:01	Outgoing HHW
Jun 06	11	.506	15:56	-.209	00:23	19:13	Outgoing HHW
Jul 06	10	.586	15:44	-.270	23:35	19:17	Outgoing HHW
Aug 06	9	.677	16:08	-.174	22:59	19:06	Outgoing HHW
Sep 06	7	.537	15:32	-.284	22:17	18:42	Outgoing HHW
Oct 06	6	.451	15:02	-.269	21:21	18:14	Outgoing HHW
Nov 06	5	.163	15:08	-.254	20:59	17:53	Outgoing LHW
Dec 06	4	.126	15:14	-.223	20:41	17:49	Outgoing LHW
Jan 07	3	.096	16:02	-.240	21:11	18:02	Outgoing LHW
Feb 07	1	.077	15:38	-.266	21:05	18:21	Outgoing LHW
Mar 07	3	.166	15:56	-.253	21:41	18:36	Outgoing LHW
Apr 07	2	.336	16:02	-.198	22:23	18:47	Outgoing HHW
May 07	2	.643	16:08	-.018	23:17	18:57	Outgoing HHW*



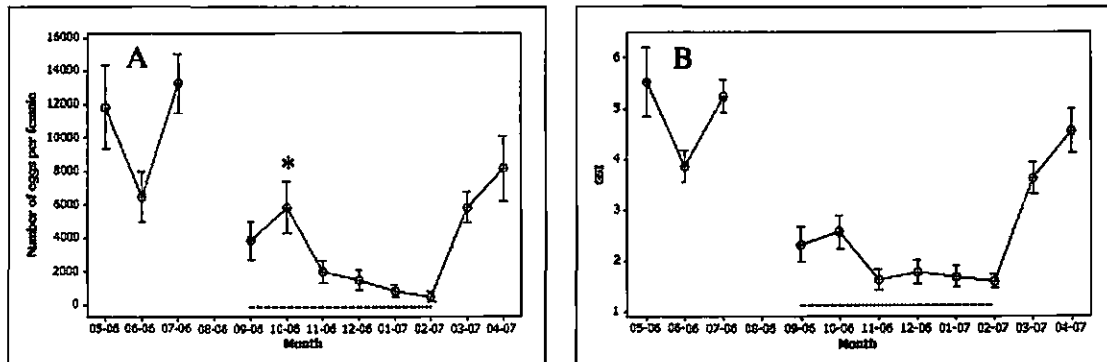


Figure 2-3. (A) Batch fecundity and (B) gonadosomatic index (GSI) of Group M female *Z. flavescens* over the year sampled (untransformed data). Bars are one standard error of the mean. Low-season months are designated by the dashed lines. All low-season months were determined to be significantly different from July 2006 data by the Tukey's test ( $p < .001$ ) except for batch fecundity in October (\*). (Tukey's test was performed on transformed batch fecundity data, as noted in text.)

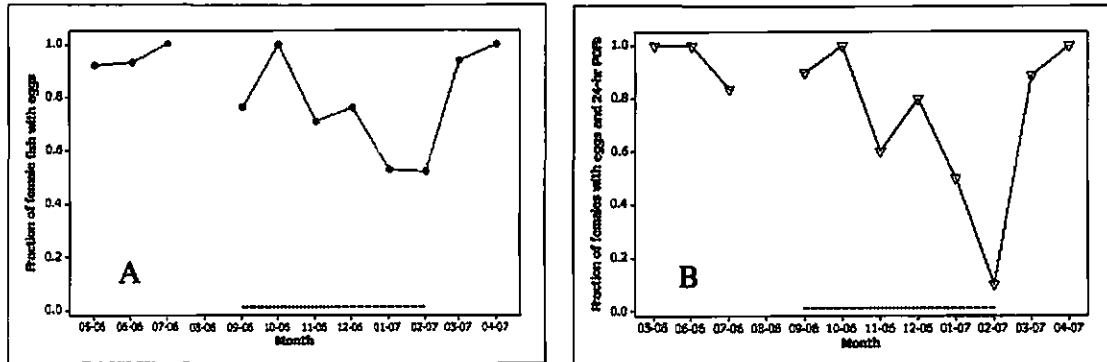


Figure 2-4. (A) The fraction of females spawning and (B) the fraction of females spawning at least two days in succession in each sample of Group M female *Z. flavescens*. Low-season months are designated by the dashed lines.

## RESULTS

### Annual season

In the Kona population, a peak in reproductive output in spring and summer months is suggested by both female GSI and batch fecundity trends throughout the sampled year (Figure 2-3). Female yellow tang do not completely cease either spawning behaviors (M.E. Bushnell, pers. obs.) or production of eggs (Figure 2-4) at any time during the year. The trends of higher GSI and batch fecundity values in Figure 2-3 in spring and summer, with lower values in fall and winter, were observed in untransformed

data. Designation of a 'high' season and 'low' season for spawning was attempted statistically, despite the lack of sampling data for August. Tukey's pairwise comparisons by month were performed for both GSI and batch fecundity data sets, and a statistical difference among months sampled was observed for both ( $p < 0.001$ , Tukey's 95% simultaneous confidence level, individual confidence level = 99.87%). Prior to the analysis, batch fecundity data were transformed to the 4<sup>th</sup> root in order to minimize unequal variances between sampled months.

The peak of reproductive output for which data are available occurred in the month of July: batch fecundity values were greatest (mean batch fecundity = 13,221 eggs), and mean female GSI was also high (5.24). Months with values not significantly different from the mean batch fecundity or GSI of July could be considered 'high' season, and those significantly different could be considered 'low' season (Figure 2-3). It is important to note, however, that the absence of data for the month of August does not permit firm, quantitative characterization of the 'season' for this calendar period of 2006.

The fraction of females appearing to be close to spawning during each month of the year was determined by establishing the percentage of females in each sample with eggs inside the ovary by means of batch fecundity count methods. Pearson Chi-Square Test (Likelihood Ratio Chi-Square = 39.422,  $df = 10$ ,  $p < .001$ ) indicates a significant difference in the fraction of female fish spawning between months, with the smallest fraction occurring in January and February 2007 (Figure 2-4A). The month of October displayed a surprisingly high percentage (100%) of spawning females. This result may be influenced by the small number of females examined in that month ( $N=9$  females). Throughout the year, the fraction of females producing eggs never decreased below 0.5, even in 'low' season months (Figure 2-4A), indicating that some level of reproduction continued throughout the year.

The fraction of the female population spawning for at least two consecutive days was determined by histological analysis of gonads from 9-12 females sampled at the

Table 2-4. The fraction of female *Z. flavescens* spawning for at least 2 consecutive days over summer 2006 (Group S fish). N = the number of females examined histologically per time point, Day/Moon = the day in relation to the full moon: 0 indicates the day of the full moon, negative numbers indicate days prior to the full moon, positive numbers indicate days after the full moon.

<i>Day/Moon</i>	<i>Day of sampling period</i>	<i>N</i>	<i>No. fish with eggs and 24-hr POFs in ovary</i>	<i>Fraction of females spawning 2 days in a row</i>
0	4	7	7	1
5	9	6	6	1
9	13	6	4	0.667
14	18	6	5	0.833
-6	28	6	6	1
-1	33	6	6	1
1	35	6	5	0.833
8	42	6	5	0.833
14	48	6	6	1
-5	58	6	5	0.833
-2	61	6	5	0.833
0	63	5	5	1

full moon of each month (Group M fish). The presence of 24-hour-old post-ovulatory follicles (POFs; see Chapter 1 for definition and description) in addition to hydrated oocytes within an ovary was taken as indication that the individual had spawned the day prior to sampling and was prepared to spawn the day of sampling, respectively. A fraction of females in every month was found to spawn on 2 consecutive days, with fewer instances of 2-day spawning suggested in the 'low' season months (Figure 2-4B). Pearson Chi-Square Test (Likelihood Ratio Chi-Square = 43.082, df = 10,  $p < 0.001$ ) indicates significant differences in the fraction of females spawning 2 days in a row between months of the sampled year.

During the intensively sampled 65-day period of May – July 2006, histological analysis of ovaries of Group S fish selected from 12 time points indicated that the fraction of females spawning at least two days in succession during the summer months remained

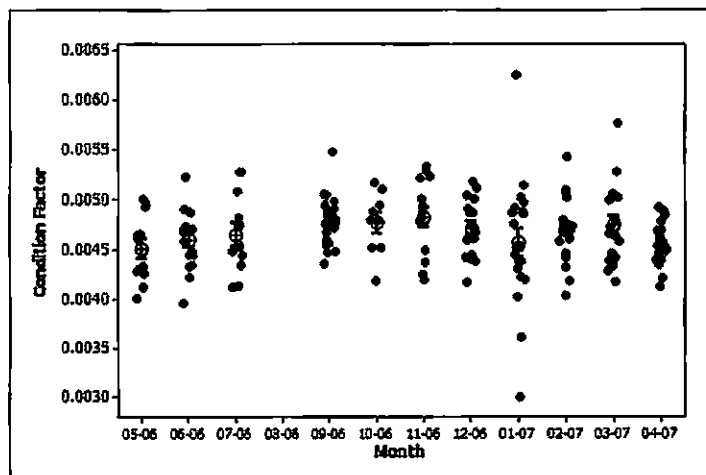


Figure 2-5. Condition factor of female *Z. flavescens* over the year sampled (Group M fish). Solid circles indicate values of individual fish, open circles indicate the mean for each monthly sample. Bars are one standard error of the mean. No significant difference in condition factor of individuals between months was established (one-way ANOVA,  $p < 0.129$ )

between 0.833 and 1.0 for all sampled days except one (Table 2-4). In contrast, the fraction of females spawning at least two days in succession for the collections of January and February - two 'low' season months - was only 0.5 and 0.1, respectively (Figure 2-4B). Therefore, the data in Table 2-4 further support the conclusion that the summer months (May-July) include a period of high reproductive effort for yellow tang females.

Female condition did not change significantly over the course of the year sampled (one-way ANOVA,  $F = 1.54$ ,  $p < 0.129$ ) as illustrated in Figure 2-5.

### ***Lunar cycle***

Lunar periodicity in spawning was suggested by the observed patterns of both batch fecundity and female GSI data during the period of intensive sampling in the summer of 2006 (batch fecundity: periodic regression,  $F = 32.5$ ,  $df = 2$ , 219,  $p < 0.001$ ; GSI: periodic regression,  $F = 38.8$ ,  $df = 2$ , 225,  $p < 0.001$ ). Peaks of mean reproductive output (i.e., batch fecundity) appeared on the full moon or within the 4 days prior to it for

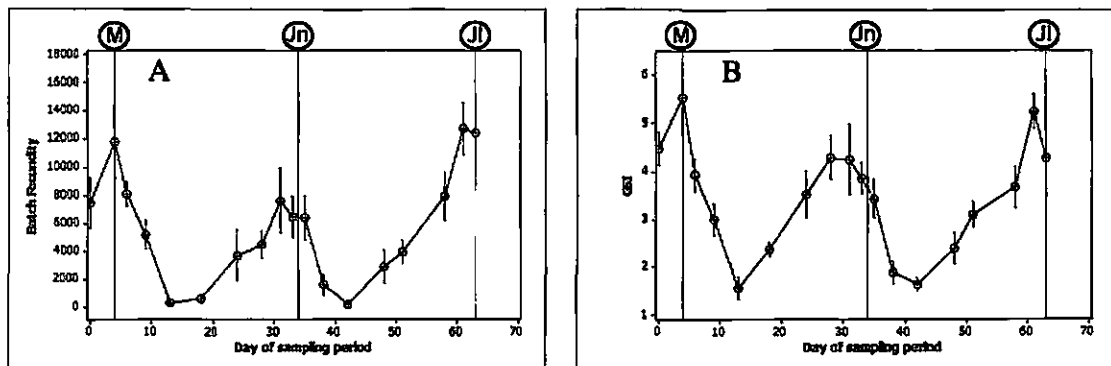


Figure 2-6. (A) Mean batch fecundity and (B) gonadosomatic index of Group S female *Z. flavescens*. Full moons of all months are indicated by lines at days 4, 34, and 63. Bars are one standard error of the mean.

each of the three full moons sampled (Figure 2-6A). Mean female GSI follows a pattern very similar to that of batch fecundity, with the highest mean GSI values appearing 2-3 days prior to the highest mean batch fecundity values for the months of June and July (Figure 2-6B). (It is likely that the peak GSI value for May was missed by inadequate frequency of sampling prior to the first full moon of the sampling period.) A brief time lag between peak GSI and peak fecundity is to be expected; the increase in GSI prior to increased spawning output is probably due to ovarian production and hydration of increasing quantities of eggs, with a resulting decrease in GSI after spawning, both of which are seen in Figure 2-6B. Providing additional support to the observed lunar cycle

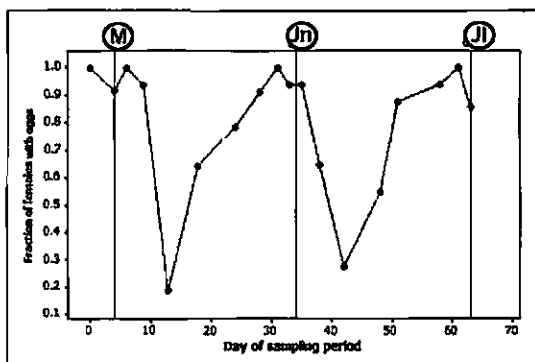


Figure 2-7. The fraction of Group S female *Z. flavescens* spawning over the summer months. Full moons of all months are indicated by lines at days 4, 34, and 63.

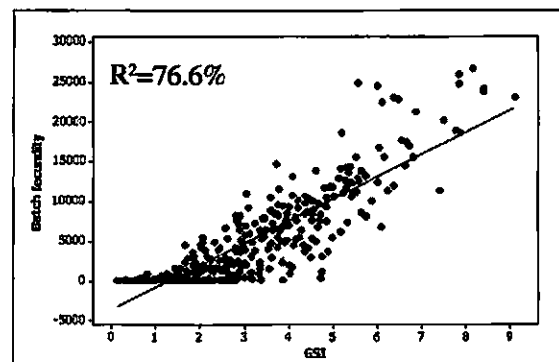


Figure 2-8. The relationship between GSI and batch fecundity of female *Z. flavescens*. Group M and Group S fish were used in the regression. Linear regression equation: batch fecundity =  $-4085 + 2997 \text{ GSI}$ ,  $p < 0.001$ .

of spawning, the fraction of females on each day sampled that contained eggs within the ovaries followed a similar lunar pattern (periodic regression,  $F = 16.5$ ,  $df = 2, 15$ ,  $p < 0.001$ , Figure 2-7).

Gonadal indices such as the GSI are often used as a proxy for measurements of fecundity; dissecting ovaries and counting eggs is a much more time-intensive process than measuring somatic and gonad weights (reviewed by West, 1990). In this species, it appears that GSI and batch fecundity of yellow tang females are closely related, with an  $R^2 = 76.6\%$  (linear regression equation: batch fecundity =  $-4085 + 2997 \text{ GSI}$ ,  $p < 0.001$ ), indicating that over 76% of the variation in batch fecundity is explained by female GSI (Figure 2-8) in all females sampled.

In order to acquire a more complete understanding of the oocyte maturation process and timing over the lunar cycle, ovaries of 5 randomly selected individual females from 11 of the 18 sampling days within the summer sampling period (Group S fish) were analyzed using histological methods. The 11 days were selected so that every quarter moon was represented, if possible. If no sampling was done on the exact quarter moon, the next closest sampling date was chosen.

The fraction of vitellogenic oocytes in each of the three vitellogenic stages (early, middle and late) was determined using size-frequency distributions of vitellogenic oocytes from cross-sectional diameters of ovaries from these selected Group S fish. A periodic cycle in egg maturation was suggested by the increase in late stage (IIIc) oocytes leading up to the May and June full moons, followed in both instances by a decrease in the percentage of late vitellogenic stage oocytes observed in ovaries at 8 and 9 days (May and June, respectively) after the full moon (Figure 2-9).

Oocyte maturation within ovaries of individual females in this population is extremely temporally variable, probably due to large differences in the number of eggs produced by individual females on any given day of the cycle. Nevertheless, periodic

regression on the fraction of oocytes in the late vitellogenic stage of all 55 females examined suggests a significant lunar periodicity of the ovarian cycle ( $F = 9.5$ ,  $df = 2, 52$ ,  $p < 0.001$ ).

Taken together, the similarity of cyclic patterns observed in batch fecundity, GSI, and late stage vitellogenic oocyte frequency strongly suggests that lunar periodicity is an inherent characteristic of the reproductive processes of this population.

### *Tidal data*

Tidal data indicate that at the time of spawning (approximately one half hour before sunset (Walsh, 1984)), outgoing tides were present on the day of the full moon in every month of the sampled year (Table 2-3).

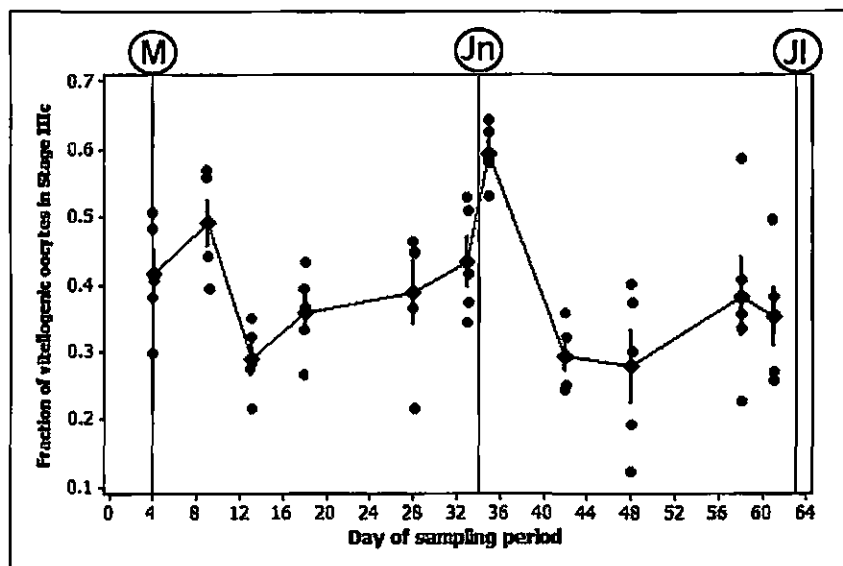


Figure 2-9. The fraction of vitellogenic stage oocytes in the late vitellogenic stage (IIIc) in ovaries of *Z. flavescens* collected over the summer months. (See Chapter 1 for descriptions of each stage). Circles indicate individual Group S fish, diamonds indicate the mean value for each day sampled. Bars are one standard error of the mean. Full moons are denoted by lines at days 4, 34 and 63.

## DISCUSSION

### *The annual reproductive season, with implications for management*

Data presented here clearly suggest year-round reproductive effort in the observed population of *Z. flavescens*, with a significant increase in egg production and spawning frequency among adult females in the spring and summer months. As this investigation covered only a consecutive 12-month period, the possibility of inter-annual variation in spawning could not be investigated. However, data collected in 2004 and 2005 from this population conform to the patterns shown in this paper (M.E. Bushnell and J.T. Claisse, unpublished data), and seasonal peaks of reproductive effort reported for populations of *Z. flavescens* on Oahu match the general trends of spring and summer reproductive intensity established here (C.W. Laidley, pers. comm.). Year-round reproductive effort may be a simple way to 'hedge bets' that environmental conditions will be advantageous for new offspring at some point during the year, if not at every spawning event (Johannes, 1978; Goodman, 1984). Additionally, without physiological constraints such as large temperature shifts in the tropics, there may be little selective pressure for *Z. flavescens* to discontinue spawning at any point in the year.

Despite the fact that egg production and spawning frequency changed significantly over the course of the sampled year, female condition did not. Condition is often associated with seasonal shifts in energy resources from somatic to gonadal growth in other marine species (e.g., Kurita *et al.*, 2003; Brown and Murphy, 2004; Gomes and Araujo, 2004; Mello and Rose, 2005). Because yellow tang females are reproductively active throughout the entire year, without a clear cessation of spawning, at which point energy may be used for somatic growth in preference to reproductive growth, maternal condition may not fluctuate as widely in this species as in some others. Continuous production of eggs throughout the year probably requires fairly constant metabolic resources, resulting in little annual change in energy expenditure. Somatic growth and maintenance would also occur at a relatively constant rate, as opposed to somatic growth



'spurts' between reproductive periods. The latter pattern has been reported in species with clearly defined on- and off- seasons of spawning (Conover, 1992).

There is still a great deal to be learned about reproduction in non food-fish tropical species. In a 2007 literature search, only a handful of papers could be found on acanthurid reproduction (for examples, see Randall, 1961a and b; Fishelson *et al.*, 1987; Colin and Clavijo, 1988; Lobel, 1989; Craig, 1998), and most studies include surgeonfish as only one of several fish families studied. Also, many of these studies were performed using small sample sizes, and all except one were completed at least twenty years ago. Importantly, the research detailed here may lead to further questions concerning the seasonality of spawning in closely related species of acanthurids in the same general location, some of which are also targeted by the Hawai'i aquarium fishery (e.g., *A. achilles*, *Ctenochaetus strigosus*, and *Naso lituratus*). It is entirely possible that these harvested surgeonfish species may also maintain a protracted spawning season, or even a year-round spawning system similar to that of yellow tang. If so, management of all these species may depend on other options as well as seasonal closure to protect reproductively active fish.

### ***The lunar cycle of spawning***

Hypotheses to explain the presence of lunar cycles of spawning are common in the scientific literature, but the actual adaptive significance of these cycles remains unclear. Possible advantages of lunar periodicity for pelagic spawning reef fishes include: synchronization of spawning with gamete release of invertebrates, thus increasing availability of food sources for new hatchlings (Thresher, 1984), synchronization of gamete release with outgoing tides to facilitate larval dispersal, or with incoming tides to minimize larval dispersal (Johannes, 1978; Ross, 1982), and synchronization of female and male reproductive behaviors, ensuring maximum reproductive effort and possibly overwhelming predators (Lobel, 1978; Thresher, 1984; Omori, 1995). As stated by Walsh

(1987) and others, no single hypothesis is likely to be 'wholly satisfactory,' although components of more than one hypothesis may contribute to the observed pattern of lunar periodicity of spawning. The relevance of the above hypotheses in relation to yellow tang spawning is addressed here.

*Zebrasoma flavescens* females release pelagic eggs in the evening. The eggs then hatch by morning of the next day, and larvae begin to feed 2-3 days post-hatch, dependent upon water temperature (S. Kraul, pers. comm.). The location of the larvae 2-3 days after spawning is largely unknown, although it can be assumed that some eggs and larvae survive within the plankton until settlement. The organisms upon which yellow tang larvae feed during the 55-day pelagic larval phase (D. Shafer, 2004) also remain largely unidentified, so knowledge concerning the spawning cycles of these planktonic prey awaits future research into pre-settlement characteristics and behaviors of this species.

As shown here, spawning activities (frequency of egg release and batch fecundity) of *Z. flavescens* increased to a peak during the 5 days surrounding the full moon. Tidal data indicate that at the time of spawning (approximately one half hour before sunset), outgoing tides were present on the day of the full moon in every month of the sampled year (Table 2-3). In months with low levels of yellow tang reproduction, November 2006 through February 2007, the outgoing tide experienced at the site was the lower of the two semidiurnal high tides, marked by smaller amplitudes than outgoing tides in late spring and summer months of April through October. Overall, it appears that the season during which reproductive effort of adult females is concentrated - late spring and summer - is also the period of time during which the outgoing tide is at the highest amplitude.

This spawning pattern may indicate a greater survival rate for larvae when off-reef dispersal of eggs due to large tidal movements is available to spawning adults. Immediate off-reef dispersal of spawned eggs has been hypothesized to aid in reduction of predation by planktivorous fishes that are more likely to be closer to the reef as light levels decrease

(Johannes, 1978; Lobel, 1978). The month of March 2007 is an anomaly in this pattern, as the lower high tide is still present at the full moon, yet female GSI and fecundity values have begun an increase denoting the beginning of the next reproductive season (Figure 2-2). However, as March is probably the start of the reproductively active portion of the 2007 year, and the tidal 'switch' between lower high tide and higher high tide in the afternoon occurs in the following month of April 2007, this may simply indicate that the general pattern of increased spawning rate during outgoing higher high tides has evolved with temporal plasticity within the population. Indeed, spawning on the outgoing higher high tide may not be the single controlling factor involved in resumption of the peak reproductive season for this species.

Finally, Korringa (1947) found an increased fertilization rate and increased adult breeding rates when marine invertebrates were entrained in a lunar cycle, and this might also be true for fish species, especially group and broadcast spawners such as *Z. flavescens*. However, underwater evening observations indicated that movement patterns of adult yellow tang do not change throughout the month, or even throughout the year (M.E. Bushnell and J.T. Claisse, unpublished data). Adults migrate from inshore feeding areas to the reef crest beginning at approximately 1 hour before sunset, and by the time of spawning, males have set up temporary territories that they patrol until dark. (For a full description of crepuscular movements and behaviors of *Z. flavescens*, see Walsh (1984)). Female fish will swim by, sometimes engaging in spawning behaviors with a male, and if a diver is careful not to disturb the fish, he/she will observe a general melee of lingering fish for the duration of the spawning period. Spawning occurs in pairs as well as in small groups of 5-10 fish. Observations of the frequency of spawning showed no clear indication that the number of spawns changed with the moon phase, although dusk observations were limited in number. Based on these limited data, the existence of a temporal peak of spawning concurrent with the full moon did not appear to affect

the breeding or fertilization rates of individuals in this population by synchronization of male and female reproductive behaviors. 'Swamping' of potential predators by the prevalence of multiple spawns at once also does not seem likely. The greatest observed 'rate' of spawning on the most active nights was about 1 spawn per minute. However, until the specific hypothesis is addressed experimentally, this explanation for lunar cycle entrainment will remain neither supported nor refuted by the current research.

### ***Implications for future research***

The observed pattern of female reproductive attributes (GSI and batch fecundity) indicates peak spawning periods around full moons of the spring and summer months for the Kona, Hawai'i Island population. The lunar pattern was observed during the 65-day intensive sampling period performed in May-July 2006. As far as the author is aware, there is no evidence to suggest that this cyclic pattern of spawning does not continue throughout all months of the year, as all other reproductive activities of females in the wild (*e.g.*, production of eggs, participation in spawning behaviors, evidence of spawning at least 2 days in succession) persisted, albeit at lower levels in winter, for all 12 months. In addition, captive fish have been shown to spawn year-round on a lunar cycle (C.W. Laidley, unpublished data). Therefore, the model created in Chapter 3 to estimate the annual reproductive potential of females in this population assumes maintenance of the lunar cycle for an entire year. Such an assumption became necessary, as continuation of the intensive sampling would have exhausted research resources as well as potentially harmed the population under investigation by removing an excessive number of reproductive females.

The lunar cycle of spawning might not have been recognized had the specific sampling protocol, which involved sampling at the same time of day in relation to sunset, not been used. Preliminary studies in this population (Figure 2-2) suggested that ovary weight, and therefore GSI, increased throughout the day due to hydration of eggs, a trend

that was also noted in *A. nigrofuscus* by Fishelson *et al.* (1987). Had daily sampling occurred within a larger window of time, GSI values between days would not have been comparable due to the confounding factor of temporal increase in ovary weight. Evidence of the lunar cycling of spawning was further complicated by the extreme variability in the number of eggs produced by individuals. On a given day in summer, the range of batch fecundity values could be as great as 0 to 28,000 eggs. In order to assess the pattern accurately, daily collections of fish had to: (1) contain a large enough sample size to limit the effects of large variation in egg production; (2) occur frequently enough so as not to miss any significant periods of the cycle; and (3) continue long enough to infer repetition of the observed pattern.

It is worth noting that the highest GSI values of females reported in the earlier Kona study (Lobel, 1989) ranged from approximately 2 to 3.5. On Oahu, C.W. Laidley (pers. comm.) measured peak GSI values of females up to 4.5. In contrast, the highest female GSI of individuals measured in the current study during summer/peak months in Kona were in the range of 4.0 to 9.1. Lobel (1989) sampled within a window of 5 hours (between 1200 hr and 1700 hr every day), which may have encompassed a significant difference in ovary weight for fish sampled at either extreme (Figure 2-2). Furthermore, Lobel's (1989) samples were taken in depths ranging from 8 to 20 m, habitat not necessarily frequented by adult schools (J.T. Claiss, unpub. data). Laidley (pers. comm.) targeted the largest adult fish available, but did not sample consistently at any specific time of day. Further, neither Lobel (1989) nor Laidley (pers. comm.) considered the effects of a lunar cycle on female GSI when sampling at either location. Taken together, these differences between sampling protocols may explain the smaller GSI values reported in past studies when compared to those in the current research. However, higher GSI of female tang in Kona, when compared to Oahu, may also be attributable to site differences.

Throughout the literature, GSI is used in almost every fish reproduction study to

distinguish reproductively active from non-reproductive fish, using the values as an index for maturity and/or fecundity. In *Z. flavescens*, a high degree of association was observed for GSI and batch fecundity in all adult females, as exhibited by the similar patterns of both over the 65-day intensive sampling period as well as parallel monthly trends over the year sampled. As the degree of association between ovarian weight, egg size, and fecundity varies greatly between fish species (West, 1990; Jons and Miranda, 1997), *Z. flavescens* may exemplify a species for which GSI serves as an acceptable indicator of batch fecundity. Thus, the time-intensive process of egg counting for fecundity measurements may not be necessary in future studies of *Z. flavescens*.

Most importantly, this research has shown that the results of an investigation of spawning that does not take into account the possibility of cyclic fluctuations of ovary weight (and therefore of GSI) over monthly or even weekly time scales may be misleading if there is reliance entirely upon GSI for reproductive characteristics of the species in question. It is entirely possible that similar spawning patterns exist in other species, especially those closely related to *Z. flavescens*, and this work should serve as a caution in the design of future studies on reef fish reproductive seasonality.

### **CHAPTER 3.**

#### **The relationship of size to fecundity and an estimate of annual reproductive output for female yellow tang (*Zebrasoma flavescens*)**

##### **ABSTRACT**

Relatively little information concerning the reproductive biology of surgeonfishes has been documented, and information of value for fisheries management remains particularly scarce. In this chapter, both a relationship between body size and fecundity and an estimate of annual reproductive output for female *Zebrasoma flavescens* (yellow tang) were sought for populations on the Kona coast of Hawai'i, where this species is the most economically important fish in the local aquarium fishery. No significant relationship between size (standard length (SL) or ovary-free weight (OFW)) and batch fecundity of adult females was found in this species (linear regression for SL,  $p < 0.086$ ,  $R^2 = 3.0\%$ ; for OFW,  $p < 0.873$ ,  $R^2 = 1.7\%$ ). The lack of a significant relationship found here for *Z. flavescens* may be due to a strongly laterally compressed body shape and small total growth as adults - the total range of size variation in adult female yellow tang was only about 5 cm. Annual reproductive output of females was estimated from a model of the cyclic pattern observed in fecundity data from summer 2006 collections. The relationship detected between batch fecundity and day of the lunar cycle was then used to estimate monthly and annual fecundity for the sampled year (approximately 1,142,000  $\pm$  142,000 eggs per female per year). Limitations of the model and the fecundity estimates are discussed.

##### **INTRODUCTION**

A positive relationship between fecundity and size (length and/or weight) of females has been established for many marine species, including *Seriphus politus* (DeMartini and Fountain, 1981), *Engraulis mordax* (Hunter *et al.* 1985), *Hemiramphus*



*brasiliensis* and *H. balao* (McBride and Thurman, 2003), *Sebastes melanops* (Bobko and Berkeley, 2004), *Gadus morhua* L and *G. morhua callarias* L (Bleil and Oeberst, 2005), and *Argyrozona argyrozona* (Brouwer and Griffiths, 2005). These and other studies have concluded that larger, and in some cases older females contribute greater numbers of eggs during the reproductive season compared to smaller or younger individuals (reviewed by Birkeland and Dayton, 2005). The aforementioned size-fecundity relationships have been established mostly for food fishes with body shapes that increase considerably in length and girth with age (*e.g.*, gadiforms and clupeiforms). No studies have addressed the relationship in surgeonfishes, which have a strongly laterally compressed body shape and a sharply asymptotic growth curve (Sadovy, 1996; Choat and Axe, 1996). One objective of the present research was to investigate the relationship between female size and batch fecundity for *Z. flavescens* - information that can be used to estimate reproductive output for the population on the Kona coast of Hawai'i Island.

An estimate of reproductive output of adult female fish is useful for managers of a population under intense fishing pressure, such as yellow tang in Hawai'i. Current regulations for the Kona coast population allow collection of this species from specific areas, but do not limit either the quantity or the size of fish taken. Conventional wisdom assumes that if larger females are responsible for a greater proportion of reproductive output than smaller females, size limits on take of the species might be beneficial for population growth and maintenance. Additionally, aquaculture programs concerned with maximizing reproductive output of *Z. flavescens* for commercial sale may find the results of this study useful.

Estimating annual fecundity for indeterminate, multiple spawning fishes is necessarily more complicated than making such estimates for determinate-spawning species with synchronous ovarian development. The ovaries of determinate species contain a fixed number of oocytes that remains unchanged throughout the breeding season up until the time of spawning (*e.g.* *Oncorhynchus* spp. and *Anguilla* spp.;

DeVlaming, 1983). This standing stock of oocytes matures simultaneously, so annual fecundity can be assessed at any point in the life cycle of the fish prior to spawning by simply counting all oocytes within the ovary (McEvoy and McEvoy, 1992); hence, the term 'determinate' spawners. In contrast, in the ovaries of multiple spawning fishes, maturing oocytes are recruited continually from primary stages, oocyte batches mature at different rates throughout the season, and the fish releases the batch of most mature stage oocytes at each spawn (Wallace and Selman, 1981). As a result, a count of the standing stock of oocytes from the ovary of an individual indeterminate spawning fish gives little or no information about the seasonal or annual fecundity of that individual. For multiple spawning species such as *Z. flavescens*, the only measurement of fecundity that is useful for such estimates is batch fecundity, as determined by counting mature, hydrated oocytes (*i.e.*, those ready to be spawned) from a fish that has been sampled just prior to the next spawning. (See Chapters 1 and 2 for spawning data.) Sampling must occur in a time window when oocytes to be released during the upcoming spawning event can be unambiguously identified, and yet at a time prior to the expulsion of the eggs (Hunter *et al.*, 1985). Measurements of batch fecundity alone are insufficient for estimating annual fecundity, however, unless the number of egg batches released by a female over the spawning period (*i.e.*, spawning frequency) can also be quantified.

Several studies have attempted to quantify spawning frequency for multiple spawning fishes (*e.g.*, DeMartini and Fountain, 1981; Hunter and Macewicz, 1985; Santiago and Sanz, 1992; McBride and Thurman, 2003; Stequert *et al.*, 2003; Roumillat and Brouwer, 2004). The most common methods involve determining the 'spawning fraction'- that is, the proportion of females in the population that are spawning - at several time points within a spawning season. Due to small sample sizes, and a sampling protocol that involved intentional sampling at assumed peak periods of reproduction throughout the year, these methods were deemed inappropriate to the estimate of reproductive output in the current study.

Instead, a mathematical model of the cyclic fluctuations observed in fecundity of yellow tang females from the 65-day intensively sampled period during the peak reproductive season, May-July 2006, was created. Using this model, the number of eggs produced per female per day in each month of the year was computed, using batch fecundity values of fish collected monthly in the year of sampling from May 2006 – April 2007 (except August 2006). The model appears to provide an estimate of fecundity that is more appropriate for yellow tang reproductive patterns than methods previously reported in the literature.

## **MATERIALS AND METHODS**

### ***Sampling***

(See Chapters 1 and 2 for detailed sampling protocols and exact numbers of fish collected per sampling date.) Fish sampled during the 65-day intensive sampling period of May 8 – July 10, 2006 were collected approximately every four days, for a total of 18 samples. Between 7 and 18 females were taken in each collection. Summer sampled fish are referred to as Group S fish. Fish sampled near-monthly for the year May 8, 2006 – April 3, 2007 were sampled on the day prior to, the day of, or the day after the full moon, and each collection consisted of between 9 and 21 females. Monthly sampled fish are referred to as Group M fish. All fish were collected less than 2 hours before sunset.

### ***Fish processing***

Batch fecundity and length and weight measurements were taken using methods described in Chapter 2.

### ***Statistical analysis***

Statistical analyses were performed using Minitab 14 software. See Chapter 2 for the basic periodic regression model used.

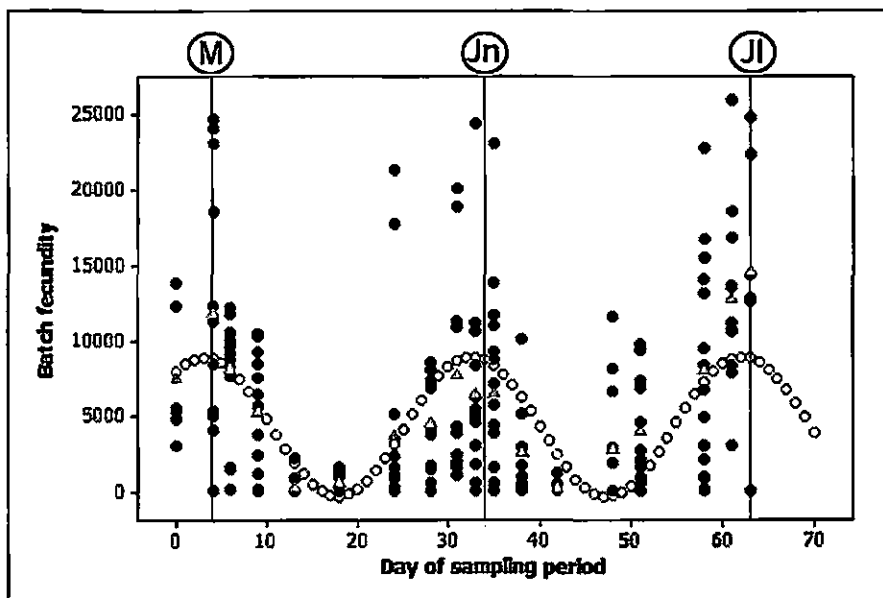


Figure 3-1. Graphical representation of the periodic regression equation from text (open circles) and batch fecundities of individual Group S female *Z. flavescens* (solid circles). Triangles indicate the mean batch fecundity value each day sampled. Full moons are indicated by lines at days 4, 34, and 63.

#### *Annual fecundity estimate*

A model of the cyclic variations in batch fecundity values observed during the 65-day summer sampling period was created to estimate daily fecundity per female for that time period. This model incorporated the equation created by the periodic regression analysis performed in Chapter 2 (from Cryer, 1986), which fitted the batch fecundity values of individual females to a sine function, as illustrated in Figure 3-1. The mean batch fecundity value for fish sampled at the full moon (or on the day prior or the day after – exact sampling dates in relation to the full moon are presented in Table 3-1) of each month of the year was then assumed to be the apex of the sine wave for that lunar month. By shifting the amplitude of the sine curve according to the monthly mean batch fecundity value (MBF), monthly egg production (MEP) was determined for 11 out of 13 months of the lunar year. (No collections were made in August 2006 or May 2007,

Table 3-1. Dates of monthly sampling of *Z. flavescens* in relation to the full moon. The full moon is denoted by a 0, the day prior to the full moon by -1, and the day after by +1. The collection for the month of December spanned two days, as previously explained in the methods section of Chapter 2. No samples were taken in August 2006. Months sampled in 2007 are underlined; months sampled in 2006 are not.

<i>Month</i>	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	<u>Jan</u>	<u>Feb</u>	<u>Mar</u>	<u>Apr</u>
<i>Day/Moon</i>	0	-1	0	-	-1	-1	-1	0/+1	0	+1	-1	0

so values for MBF and MEP were interpolated for August based on the midpoint values between July and September 2006, and for May 2007 based on the midpoint values between April 2007 and the previous year's May 2006.)

From Chapter 2, periodic regression data for fecundity over the summer sampling period gave the equation:

$$Y = 4274 + 3673(\cos\theta) + 2800(\sin\theta) \quad (\text{EQUATION 3-1})$$

Where the y-intercept, 4274 eggs, is the mean number of eggs produced by one female per day over the 65-day sampling period. The y-intercept is the midpoint of the sine wave, and the relationship between the Midpoint (M), Apex (A), and Trough (T) of the wave must remain constant as the wave is transposed for each of 13 lunar months, with different values for the apex (and hence the midpoint) determined by mean fecundity values from samples in each month. Because the trough (the lowest number of eggs produced per female per day) for each month cannot be negative, it is assumed to be zero for every month, since it was approximately zero at the peak of the reproductive season.

For example, for the summer months, the equation states:

$$M = 4274 \text{ eggs}$$

$$T = 4274 - \sqrt{3673^2 + 2800^2} = -344 \text{ eggs} \quad (\text{EQUATIONS 3-2A, B, AND C})$$

$$A = 4274 + \sqrt{3673^2 + 2800^2} = 8892 \text{ eggs}$$

Where M is the midpoint, T is the trough, and A is the apex/peak of the sine curve for the model. Having a negative number of eggs (T = -344 eggs) is unrealistic, so the trough is set to zero for the sake of estimation. The relationship between peak (8892) and

midpoint (4274) of the model curve is essentially a 2:1 ratio, which is derived from the definition of a sine function.

Based on this relationship, the apex value of each month (the average monthly collection fecundity measurement from Group M fish, or MBF), divided by 2 (as per the sine wave function representation) and multiplied by 28 days in the lunar month, results in an estimate for the average number of eggs produced per female for that lunar month, *i.e.*:

$$MEP_n = \frac{MBF_n}{2} \times 28 \quad \text{OR} \quad MEP_n = MBF_n \times 14 \quad (\text{EQUATIONS 3-3A AND B})$$

Once the MEP for all months was calculated, annual fecundity (AF) was computed using the equation:

$$AF = \sum_{1}^{13} MEP_n \quad (\text{EQUATION 3-4})$$

Variance of the estimate, or  $\sigma^2$ , was computed as the sum of the variances of the monthly estimates (the standard error of each month, or MSE), using the following equation:

$$\sigma^2 = 14^2 \times \sum_{1}^{13} (MSE_n)^2 \quad (\text{EQUATION 3-5})$$

## RESULTS

### *Size-fecundity relationship*

An extraordinary amount of variation in batch fecundity values of females of all sizes was observed in this population. Adult female yellow tang exhibited no significant relationship between either standard length (SL) or ovary-free weight (OFW) and batch fecundity (BF) (N=169 fish, SL linear regression equation:  $BF = -14693 + 154 SL$ ,  $p < 0.086$ ,  $R^2 = 3.0\%$ ; OFW linear regression equation:  $BF = 426 + 45.6 OFW$ ,  $p < 0.873$ ,  $R^2 = 1.7\%$ ). Both relationships are illustrated in Figure 3-2. Only Group M fish were used in the regressions.

The smallest female encountered with eggs inside the ovary (with a total batch fecundity count of 919 eggs) was 103 mm SL, as illustrated in Figure 3-2A. Data from Figure 3-2A also indicate that female fish over 118 mm SL are capable of producing at least 5000 eggs per batch.

The difference in SL between the smallest (103 mm) and largest (156 mm) adult female measured in this study (N=490) was small - 53 mm. The largest adult from Group M (N=169) was 144 mm SL (Figure 3-2A).

Batch fecundity of Group M females varied across months, as shown in Chapter 2, but standard lengths did not (One-way ANOVA,  $p < 0.893$ ) as illustrated in Figure 3-3.

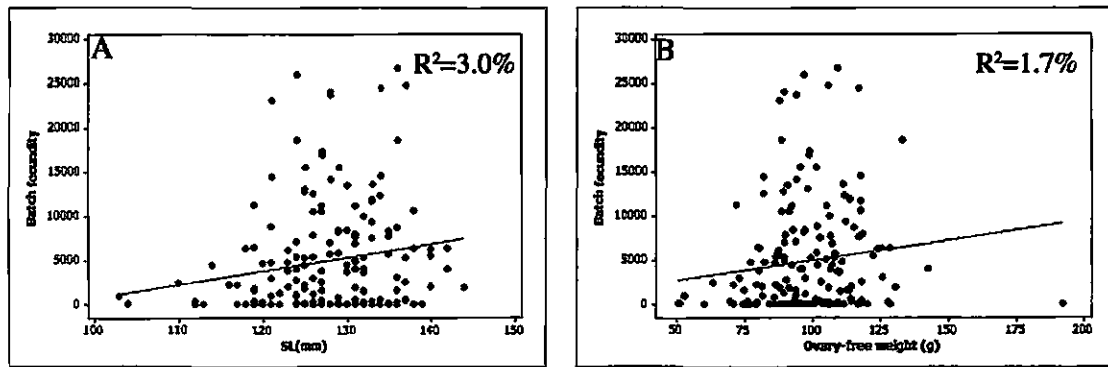


Figure 3-2. Relationship of batch fecundity to (A) standard length (linear regression equation:  $BF = -14693 + 154SL$ ,  $p < 0.086$ ) and (B) ovary-free weight (linear regression equation:  $BF = 426 + 45.6OFW$ ,  $p < 0.873$ ) in Group M female *Z. flavescens*.

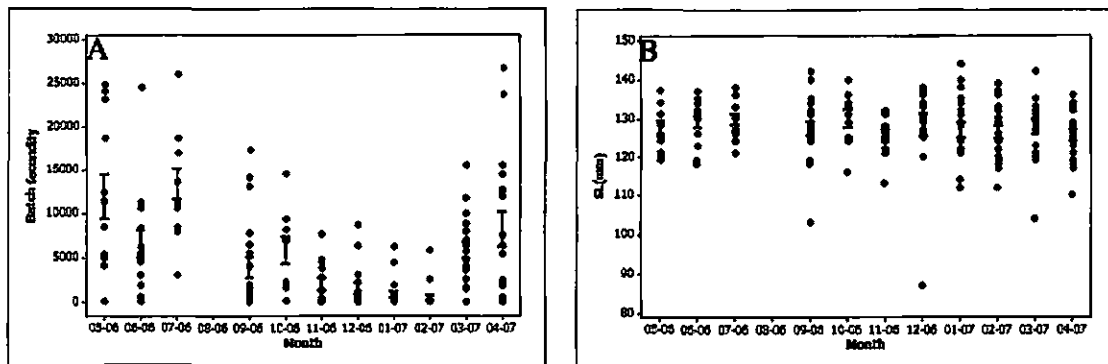


Figure 3-3. (A) batch fecundity and (B) standard length of individual Group M female *Z. flavescens* (solid circles). Batch fecundity varied significantly across months (from Chapter 2, Tukey's pairwise comparison  $p < 0.001$ ), but standard lengths did not (one-way ANOVA,  $p < 0.893$ ). Bars indicate one standard error of the mean.

Table 3-2. Monthly and annual fecundity estimates of *Z. flavescens*. MBF = monthly mean batch fecundity, MEP = monthly egg production, MSE = Monthly standard error, Model SE = model standard error, AF = annual fecundity, or number of eggs produced per female per year. MBF, MEP, and MSE values were interpolated for the months of August 2006 and May 2007 due to lack of sampling data.

<i>Month</i>	<i>N</i>	<i>MBF</i>	<i>MBF /2</i>	<i>MEP</i>	<i>MSE</i>	<i>Model SE</i>
May-06	12	11,804	5902	165,256	2525	
Jun-06	15	6504	3252	91,056	1580	
Jul-06	7	12,445	6223	174,230	3669	
Aug-06	-	8381	4190	117,327	5604	
Sep-06	17	4316	2158	60,424	1358	
Oct-06	9	5816	2908	81,424	1561	
Nov-06	14	3239	1620	45,346	1345	
Dec-06	17	2741	1371	38,374	1125	
Jan-07	19	1271	636	17,794	725	
Feb-07	21	1113	557	15,582	772	
Mar-07	18	5802	2901	81,228	950	
Apr-07	18	8136	4068	113,904	1931	
May-07	-	9970	4985	139,580	5992	
<b>AF</b>	<b>167</b>			<b>1,141,525</b>		<b>±141,910</b>

#### *Annual fecundity estimates*

In previous studies (e.g., DeMartini and Fountain, 1981; Hunter and Macewicz, 1985; Santiago and Sanz, 1992; McBride and Thurman, 2003; Stequert *et al.*, 2003; Roumillat and Brouwer, 2004), estimates of MEP were calculated for each of several size classes of female fish based on a positive relationship of fecundity and total length or weight. Since yellow tang females clearly displayed no relationship between fecundity and length or weight (Figure 3-2) in this study, MEP values were calculated from each sample of adult females as a whole, with no distinction made between females of different size classes. Annual fecundity calculated from the model was estimated to be about 1,142,000 ±142,000 eggs per yellow tang female per year (Table 3-2).



## DISCUSSION

### *Size-fecundity relationship*

A significant relationship between size and batch fecundity was not observed in *Z. flavescens*, in contrast to many other perciform species (e.g., DeMartini and Fountain, 1981; Davis and West, 1993; Roumillat and Brouwer, 2004; Brouwer and Griffiths, 2005). It is possible that positive size-fecundity relationships are not typical for fish with strongly laterally compressed body shape and non-standard logarithmic growth patterns. Instead, many surgeonfishes grow to near maximum length within a relatively short period of time, and then growth essentially stops (Choat and Axe, 1996). Furthermore, the total range of variation in maximum size of female yellow tang is very small; the difference in length between the smallest and largest adult female measured in this study (N=490) was only 5.3 cm. Constraints on body size, in terms of length, width, and depth, may restrict ovaries from growing past a maximal size, with the obvious result that the number of eggs produced is limited by space within the body cavity. For fully mature female *Z. flavescens*, it appears that batch fecundity varies not with length or weight of the fish, but with other factors, such as time of month or season, as shown previously in Chapter 2.

One common goal of fisheries biologists is to estimate the Size of First Reproduction (SFR) for harvested fish species, which is then typically used to create management strategies with an aim to protect spawning females. Figure 3-2A suggests that the smallest female to produce eggs is about 103 mm SL, and that any female fish over 118 mm SL is capable of spawning at least 5000 eggs per batch (approximately 20% of the largest observed batch). Given the small sample size of females less than 120 mm SL in this data set, a precise determination of the point at which 50% of the population is producing eggs - a common definition of SFR (Sadovy, 1996) - would not be feasible, since all size ranges of females were not sampled equally. Instead, a description of the data which notes that 118 mm SL appears to be the length at which females provide a

substantial number of gametes to the population seems to be more informative, based on the data available.

It is important to note that this study addressed only the possible relationship between batch fecundity and size of females at peak spawning times (around the full moon). This investigation did not address whether larger females spawn more frequently or over a more protracted spawning season, or whether they contribute higher quality eggs - all characteristics that have been shown to relate positively with body size in some fish of other species (*e.g.*, Bagenal, 1971; DeMartini and Fountain, 1981; Lambert, 1987; Quinn *et al.*, 1995; Trippel and Neil, 2004; Abdoli *et al.*, 2005; Birkeland and Dayton, 2005).

This study also found large variability in the number of eggs produced by females of all sizes, especially in the spring and summer. However, factors responsible for this variability remain unknown. Production of different-size batches of eggs may follow a pattern that was not readily discerned by the sampling method. For example, a female may spawn for 5 days in succession, with a large batch on day 3, but smaller batches on days 1, 2, 4 and 5. Larger females may exhibit patterns in spawning batch sizes that differ from the patterns of smaller individuals. Observation of spawning habits of individual fish over time are needed to suggest why some females spawn more eggs than others on a given day of the lunar cycle or year.

### ***Annual fecundity estimates***

#### ***Previous fecundity estimation methodology and its limitations for this study***

Most estimates of reproductive potential in multiple spawning fishes have been calculated using a variation on one of two methods: the hydrated oocyte method (DeMartini and Fountain, 1981) and the POF method (Hunter and Macewicz, 1985). Both methods rely upon estimating spawning frequency of a population using either the presence/absence of eggs (hydrated oocyte method) or the presence/absence of

histological indicators of prior spawning in the ovary (POF method). The fraction of females spawning within the population is compiled from fish captured on sampling days throughout the season. Spawning frequency is then multiplied by the average number of eggs produced per female per spawn to get an estimate of the number of eggs produced over the course of the spawning season or year. In studies such as these, sampling is performed at intermittently spaced time intervals, with the intent to sample as often as possible but not necessarily at specific times in the spawning cycle.

In the current investigation, sampling was conducted only once per month (minus August) for one calendar year, and it was performed intentionally at the time of assumed peak reproductive effort for that month (on or near the full moon). Any calculations of spawning frequency and of reproductive output based on these samples would therefore overestimate reproductive output, if applied uniformly for the period of time investigated.

Furthermore, had the hydrated oocyte method been used in the current study, a calculation of frequency of female spawning in off-season months could be misleading. (The POF method was also not ideal, as it requires expensive histological processing.) A female fish would have to be considered as part of the spawning fraction as long as at least one hydrated oocyte or ovulated egg was present in the ovary at the time of capture. According to the method, at any point in the year, a fish with 20 eggs in her ovary would be considered an equal part of the spawning fraction as a female with 20,000 eggs. This would hold true for calculations of spawning frequency in all months, but occurrence of females spawning less than 100 eggs was high only in low-season months. (During the high-season, only 4.3% of females had between 1 and 100 eggs in the ovary; during low-season months, 11.1% of females had between 1 and 100 eggs.) The question of whether these fish that spawn so few eggs at a time do contribute substantially to the reproductive potential of the population would add further uncertainty to an estimate of egg production that relied upon spawning frequency calculated in this way. Use of the periodic regression model, however, does not distinguish between spawning and non-spawning females, as it

takes into account only the number of eggs produced on a given day of the cycle by the average female in the population.

*Periodic regression model and its assumptions*

These considerations suggest that, given the type of data available, using periodic regression to model the lunar cycle of spawning produces a more accurate estimate of annual fecundity than would be obtained by previously described methods. This is because the periodic regression model incorporates the lunar periodicity observed in batch fecundity of summer months into daily fecundity estimates for females year-round.

Two major assumptions are employed in this model: (1) that the lunar periodicity of spawning observed in the summer months continues in all months of the year, and (2) that the cyclic pattern of spawning is adequately represented by a sine wave. Neither of these assumptions is trivial. The first assumption seems reasonable and consistent with all present data, including that of spawning cycles observed in captive fish (C.W. Laidley, unpublished data), and cannot be tested without extensive additional field sampling, so it is provisionally accepted for the purposes of this estimate. To address concerns about the use of the sine curve to approximate the change in fecundity values throughout the months, some of the major sources of error with the use of the model should be noted.

First, a discrepancy exists between the peak fecundity of the model (which appears on the day prior to the full moon of each month, Figure 3-1) and the assumed 'peak' of reproduction from each month, which was sampled on either the day prior to, the day of, or the day after the full moon. In fact, considerable effort was made to perform monthly collections on the day prior to the full moon (Table 3-1), and only when sampling proved impossible due to weather conditions was sampling performed on other days of the lunar month. Because the model serves to represent the trend observed in average batch fecundities of females, this discrepancy is worth noting, but is considered acceptable for the use of the model. The failure of the sampling dates to overlap exactly

with the model peaks may result in slight over- or underestimation of fecundity for a given month, but given the large standard error reported for the total estimate, it is not likely to provide much uncertainty to the overall estimate.

It should not be assumed that the day prior to the full moon of every month represents precisely the peak of reproductive effort for all females in the population, although this time-point was derived from the periodic regression equation, and sampling was conducted accordingly. Information concerning the time of peak reproductive output of this species is not precise, and the best estimate of timing of the monthly peak of yellow tang egg production may be that it is 'likely to be' within the 3 days prior to and the day of the full moon (Chapter 2).

Further assumptions were made so as to make the sine function better represent the temporal spawning patterns. The trough of the estimate was assumed to be zero in every month, instead of using the model's sine function value, which computed a negative number for egg production at the trough. Also, the sine function created a broader-peaked shape than the trend in the mean batch fecundity values in the summer (Figure 3-1). For example, in May and July, the data peaked more sharply (and at higher y-values) than the sine curve, which underestimated peak values for those months.

Finally, the inclusion of interpolated MBF and MEP values for 2 of the 13 months analyzed (August 2006 and May 2007) adds further uncertainty to the accuracy of the estimate. Confidence in a number created from this model is at least as limited as the confidence interval presented, which in this case is about  $\pm 142,000$  eggs/year, or about 12% of the estimated total egg production. The estimate of variance of the model probably underestimates the uncertainty of annual egg production, because the computation of the model's standard error (Equation 3-5) assumes that the multiplication by 14, based on the sine curve function (from Equation 3-3), does not harbor intrinsic error. Figure 3-2, as well as the preceding paragraph, clearly shows that 'intrinsic error' is present in the sine wave function model. Nevertheless, the model seems to provide

a more realistic estimate of annual fecundity than would the hydrated oocyte or POF methods, given the cyclic fluctuations in spawning activities and the limitations of the collected data.

The results developed and presented in this chapter are intended to provide researchers and managers with a starting point – a “first cut” estimate of the number of eggs the average adult female *Z. flavescens* produces in one year. More accurate estimation of the annual fecundity of this species would require significantly increased sample sizes in addition to identification of the currently unknown variables responsible for the large variation in batch fecundity among individual female tang sampled on the same day.

## SUMMARY AND CONCLUSIONS

This thesis represents the most comprehensive investigation of *Zebrasoma flavescens* reproduction to date. Information concerning the production cycle of egg maturation, timing of spawning, fecundity, and seasonality of spawning presented herein provides researchers with an understanding of the basic reproductive biology of this surgeonfish species. This information will further contribute to the scientific pursuit of knowledge of reproduction in other multiple spawning fishes in the marine tropics. Specific conclusions resulting from the completion of this work are highlighted below.

### *Histological investigation of ovaries*

Information obtained through histological investigation of this species proved fundamental to the successful execution of this study. Microscopic analysis of ovaries revealed that *Z. flavescens* is a multiple spawning species with indeterminate fecundity characterized by asynchronous development of oocytes. The prevalence of females spawning for at least two consecutive days was documented, and confirmation of a diel pattern of spawning was achieved. Without specific insights into the timing and frequency of spawning of female tang, the investigation of spawning seasonality in this species would have been much less informative.

Based on the findings presented here, histological investigation of ovarian composition and oocyte maturation is suggested for studies of related acanthurid fishes prior to investigation of other aspects of their reproduction. Differences in timing of egg production, spawning behaviors, spawning seasonality and presence/absence of lunar cycles have been observed in the few acanthurid species previously studied (Randall, 1961a, 1961b; Fishelson *et al.*, 1987; Colin and Clavijo, 1988; Fouda *et al.*, 1988). This thesis offers support for the use of histology as the most accurate tool for determining

spawning patterns of multiple-spawning females, and the evidence presented indicates that histology should be at least considered as an essential first step in the investigation of egg production in most indeterminate spawning fish species.

#### ***Zebrasoma flavescens* spawning**

A protracted spawning season was observed for *Z. flavescens* in the Kona population, with peak reproductive effort during the spring and summer months. A lunar cycle of spawning was also detected, with an increase in the number of eggs produced and the number of spawning events occurring around the full moon.

The studied population exhibited a high degree of variability in the size of egg batches, which was not explained by either length or weight of females. However, variability in batch size was partially associated with temporal changes in relation to the moon phase. Diel and lunar spawning patterns had been masked by sampling methodologies used in earlier studies which did not recognize either daily or monthly cycles of spawning in other locations (Lobel 1989; C.W. Laidley, pers. comm.). It is likely that some variables contributing to differences in individual spawning frequency and fecundity were missed in the current investigation. More work is necessary to fully understand the major variables responsible for differing levels of reproductive output between females of this species.

#### ***The estimate of annual reproductive output for female Zebrasoma flavescens***

This is the first study to investigate annual fecundity of *Z. flavescens*, and in fact, one of only two studies to investigate fecundity of any member of the family Acanthuridae. The previous investigation of *Ctenochaetus striatus* by Fouda *et al.* (1998) reported fecundity ranging from 46,963 to 575,448 eggs, with oocyte size frequency distributions of females suggesting 'continuous egg production.' Unfortunately, no definition of 'fecundity' appears in the paper. (The methods section states that the



number of 'eggs' per female was extrapolated from weighted subsamples of ovaries of 23 individuals, but the 'eggs' counted were not explicitly defined, *e.g.*, as mature or hydrated oocytes.) The fecundity measurement reported by Fouda *et al.* (1998) could very well be the number of oocytes of all stages found within the ovary upon dissection. As shown in Chapter 2 and discussed in Chapter 4 of the present thesis, this gives little information as to the realized seasonal or annual fecundity of a multiple-spawning fish. It seems equally likely that the fecundity values reported by Fouda *et al.* (1998) may, in fact, refer to a count of fully mature eggs. Unfortunately, without an estimate of spawning frequency for *C. striatus*, the relationship between Fouda *et al.*'s (1998) reported fecundity and the realized annual fecundity of the species is difficult to interpret and probably not usable in any practical management context. Furthermore, Fouda *et al.*'s (1998) measurement of fecundity for *C. striatus* is not comparable to the annual fecundity values reported here for the closely related species *Z. flavescens*. It is clear that the concept of "fecundity" must be carefully defined in each study, in order that the results be interpreted correctly by interested scientists.

In the present study, estimation of total annual fecundity of *Z. flavescens*, a multiple-spawning fish, required use of a mathematical model to replicate the cyclic pattern observed in batch fecundity data of summer sampled fish. The model estimated fecundity of the individual yellow tang female to be  $1,141,525 \pm 141,910$  eggs per year. It is the intent of this work that this reproductive estimate of *Z. flavescens* be used in a management context for the location studied, and also that the method of estimation of annual fecundity be considered for use in the investigation of egg production for other populations of *Z. flavescens*, and for similarly reproducing species.

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